Molecular species of phospholipids in a murine stem-cell line responsive to erythropoietin

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The generation of the lipid signalling molecules, diacylglycerol (DAG) and phosphatidic acid (PA), has been implicated in the transduction events essential for proliferation of murine B6Sut.EP stem cells responsive to erythropoietin (EPO). Some of the responses were rapid and transient while others were slower and sustained. In an attempt to better understand the biphasic nature of DAG and PA appearance in response to EPO, an analysis of the molecular species of DAG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and PA in control and EPO-treated B6Sut.EP cells was made by HPLC and TLC. Fifteen to eighteen species were identified, which were increased non-uniformly by 0.2 unit/ml EPO. Greater increases ($\times 6$) were observed in 16:0,20:4 and 18:0,20:4 DAGs than in other species. The molecular species profiles of the

stimulated DAGs were compared with the profiles of molecular species contained in the phospholipids. Comparison of the increase in DAG species caused by EPO with the molecular species present in PC and PI showed both PI and PC as the source of the fast DAG accumulation and only PC as the source of the slow DAG accumulation. PE was involved in both phases. We found a consistent formation of ethanolamine over time, in larger amounts than choline, providing strong evidence that, in addition to PC, PE is a major substrate. In addition, changes in molecular species of PA in response to EPO suggest that PI cannot account for the mass of PA formed during the first 30 s incubation with EPO, nor for PA formed during 30 min with EPO. It is concluded that the majority of PA was formed by a direct action of phospholipase D on PC.

INTRODUCTION

Haematopoietic growth factors, such as erythropoietin (EPO), stimulate a series of signal transduction events in order to affect proliferation and differentiation of their target cells. In a companion paper [1], we have identified early and late changes in diacylglycerol (DAG) and phosphatidic acid (PA) accumulation linked to G-protein-coupled phospholipase D activation. The identification of the molecular species of the DAGs and PA in response to EPO is important in establishing the source and potential role of these lipid signalling molecules.

Since earlier studies from this laboratory and others [2–4] have implicated protein kinase C (PKC) as an essential mediator of the mechanism of action of EPO and because phosphatidylcholine (PC) hydrolysis produces species of DAG that are effective activators of PKC *in vitro* [5], it is important to establish which species of DAG are produced in response to EPO. Specific PKC isoforms may be differentially sensitive to molecular species of DAG. The physiological role(s) for PA are still not well understood but many have been proposed, including stimulation of calcium influx, inhibition of adenylate cyclase, stimulation of DNA synthesis and inhibition of GTPase-activating protein (GAP) activity [6–11].

Several studies have identified phosphatidylethanolamine (PE) as a lipid which is elevated in neoplastic cells [12,13]. Lacal et al. [12] found that in *ras*-transformed cells, DAG was elevated and PC and PE were the major sources of DAG. Purified phospholipase D recognized either PC or PE as a substrate. The K_m values were 0.74 mM and 0.91 mM respectively [14]. If the DAG produced by cells treated with EPO is being generated by hydrolysis of a given phospholipid, then the molecular species profile of DAG should be nearly identical to that of the hydrolysed phospholipid. Thus, it is possible to discern the

source of stimulated DAGs by comparing the molecular species profiles of the cellular DAGs with those of phospholipids. In this study we present an analysis of the molecular species of DAG, PC, PE, phosphatidylinositol (PI), and PA in control and EPO-treated B6Sut.EP cells. The results suggest that hydrolysis of PI and PE contributed most of the DAGs and PA generated at early times, whereas PC and PE hydrolysis contributed the majority of DAGs at later times.

MATERIALS AND METHODS

Materials

Media and components for B6Sut.EP cells were obtained from GIBCO. Tissue-culture plasticware was obtained from Corning (Corning, NY, U.S.A.). Solvents were from Mallinckrodt Speciality Chemicals (Paris, KY, U.S.A.) and standards for HPLC from Alltech Associates (Deerfield, IL, U.S.A.). Hydrofluor counting mixtures were obtained from National Diagnostics (Somerville, NJ, U.S.A.). Lipid and fatty acid standards, including plasmalogens, were from Serdary Research Laboratory (Ontario, Canada) and Avanti Polar Lipids, Inc. (Birmingham, AL, U.S.A.). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Highly purified recombinant EPO was obtained from McDonnell Douglas (St. Louis, MO, U.S.A.).

Preparation of cells

B6Sut.EP cells are a subclone [15] of a previously established murine bone-marrow stem-cell line B6Sut.A [16], which were

Abbreviations used: DAG, diacylglycerol; EPO, erythropoietin; GAP, GTPase-activating protein; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PS, phosphatidylserine; RRT, relative retention time.

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Table 1 Distribution of fatty acid molecular species of DAG and phospholipids in cultures of B6Sut.EP cells before addition of EPO

The major fatty acid species were determined as described in the Materials and methods section. Each molecular species was numbered based upon its retention time. RRT was calculated with respect to 16:0,22:6 (peak 9). The actual DAG molecular species represented by each peak was determined by comparison either with the RRT of standard DAG or with the predicted RRT calculated as described in the Materials and methods section. The S.D. of RRT of DAG standards were ± 0.001 ; for DAG S.D. = ± 0.2 – 1.2% ; for PC, PI and PE S.D. = ± 0.1 – 0.6% ; and for PA S.D. = ± 0.1 – 0.7% .

Peak no.	<i>sn</i> -1, <i>sn</i> -2 molecular species	RRT	Molecular species (% of total)				
			DAG	PC	PI	PE	PA
1	14:0,22:6	0.579	0.38	0.62	0.29	0.33	—
2	18:2,18:3 and 14:0,20:4	0.621	0.60	0.58	0.28	0.16	—
3	16:1,18:2	0.687	0.83	0.96	0.30	0.38	—
4	18:2,22:6 and 15:0,22:6	0.759	0.74	1.09	0.56	0.22	0.38
5	16:0,20:5 and 18:2,20:4	0.791	1.52	2.32	0.42	1.72	—
6	18:2,18:2	0.834	0.88	1.28	0.49	0.58	—
7	16:0,16:1	0.895	0.33	0.74	0.61	0.38	—
8	16:1,18:1	0.972	0.86	0.88	0.55	0.56	0.50
9	16:0,22:6	1.000	2.62	3.96	4.10	9.26	1.95
10	16:0,20:4	1.059	11.70	9.69	0.40	10.55	2.36
11	16:0,18:2	1.117	13.62	15.34	4.19	12.26	2.68
12	18:1,18:2	1.156	1.63	3.44	0.92	6.08	1.46
13	16:0,22:5 (<i>n</i> –3)	1.317	0.33	1.04	0.70	1.13	0.62
14	16:0,22:5 (<i>n</i> –6) and 18:0,20:5	1.355	0.54	1.70	0.69	1.00	1.48
15	16:0,20:3 (<i>n</i> –6)	1.432	0.79	1.40	0.99	0.51	1.32
16	17:0,18:2 and 16:0,20:3 (<i>n</i> –9)	1.470	0.88	1.22	0.85	0.63	0.55
17	16:0,18:1	1.549	1.10	1.64	1.06	0.97	0.40
18	18:1,18:1	1.644	4.00	6.38	2.09	2.10	0.36
19	18:0,22:6	1.738	3.20	3.30	3.18	8.36	24.54
20	18:0,20:4	1.841	34.80	16.99	59.03	28.96	38.93
21	18:0,18:2	1.941	10.00	14.89	2.18	6.79	5.20
22	18:0,18:1	2.066	1.58	1.06	2.28	2.46	5.28
23	18:0,22:5 (<i>n</i> –3)	2.251	1.40	3.51	1.08	0.98	1.44
24	18:0,22:5 (<i>n</i> –6)	2.355	0.93	1.71	2.65	1.32	2.31
25	18:0,20:3 (<i>n</i> –6)	2.495	2.02	1.24	2.49	0.98	1.68
26	18:0,20:3 and 18:1,20:4	2.679	1.42	2.02	5.72	1.32	4.88
27	20:0,22:6 and 20:0,20:4	2.785	1.30	—	2.08	—	1.68

isolated following growth in soft agar in the presence of EPO. These cells were maintained in RPMI-1640 medium supplemented with 15% (v/v) fetal bovine serum, 10^{-5} M 2-mercaptoethanol, 2% (w/v) minimal essential medium vitamins, 2% (w/v) non-essential amino acids, 1% penicillin/streptomycin, 1% fungizone, 1% L-glutamine and 5% (v/v) conditioned medium from WEHI-3 cells.

Determination of DAG levels

Cells were incubated with EPO (0.20 unit/ml) for 0–100 min, depending on the experiment. Cellular lipids and the mass of DAG were determined as previously described [1].

TLC and HPLC of PA were performed as described in the accompanying paper [1].

Analysis of molecular species of DAG, PA and phospholipids

Extracts of DAG were incubated with 3,5-dinitrobenzoyl chloride in dry pyridine at 60 °C for 10 min, and the mixture was extracted with n-hexane as described by Kito et al. [17]. Butylated hydroxytoluene (10 µg) was added to the 2 ml hexane extracts for storage under N₂ prior to HPLC. HPLC was performed using a Waters model 510 solvent delivery system with a model 450 absorbance detector set at 254 nm and a Beckman Ultrasphere 5-mm Octyl column (4.6 mm × 25 cm). The mobile phase, acetonitrile/propan-2-ol (80:20, v/v), was degassed at a flow rate of 1 ml/min. Samples were dried under N₂ and dissolved in 200 µl of acetonitrile for injection. The detector output was

channelled through an analog/digital convertor and stored in an LIS Sunquest computer. Integration of peaks was performed using the Hewlett-Packard CPlot system. Peaks were identified when possible by reference to published relative retention times (RRTs) [18] using a 16:0, 22:6 standard. Assignments of peaks (major) were confirmed by performing fatty acid analysis as described previously [18]. Two methods were used: HPLC of fatty acids converted into 2-nitrophenyl hydrazides, or GC of transmethylated fatty acids with BF₃/methanol. The two methods gave the same fatty acid composition for most of the peaks. The major fatty acid peaks (designated by numbers) with their RRTs are given in Table 1.

Phospholipids [PC, PE, phosphatidylserine (PS), PI and PA] were separated and purified as described previously [1]. PA was hydrolysed to DAG by alkaline phosphatase and HPLC performed as described by Lee et al. [19]. Distearoyl PA (1 nmol/mg of cell protein) was added as an internal standard to the PA lipid extract, and the mixture was dispersed by sonication in 50 mM Tris/HCl buffer and then incubated with calf intestine alkaline phosphatase (10 units) as described by Ehle et al. [20]. The DAG produced was benzoylelated as described above, and the molecular species analysed as for benzoylelated DAG. This method of analysis of molecular species of PA is simple and may be used in routine determinations. The method compared well for the majority of molecular species of PA with reverse-phase ion-pair HPLC developed by Abidi [21] in which the mobile phase was acetonitrile/methanol/water (70:28:8, by vol.) containing tetrabutyl ammonium phosphate (5.0 mM) on a stationary phase of

Adsorbosphere HSC₁₈ (25 cm × 4.6 mm; internal diam. 5 µm). The major discrepancy was the 16:0, 20:4 pair, which was 2.30 % with the described method but 0.1 % by the Abidi method. Both methodologies slant the detection towards less saturated PAs. As quantitative hydrolysis by alkaline phosphatase is not complete for some more saturated molecular species, our results may overestimate polyunsaturated species while underestimating the more saturated species.

PC, PE, PI and PS were separated into molecular species on a 4.6 mm × 250 mm Ultrasphere ODS column (Altex Scientific, Inc., Berkeley, CA, U.S.A.). PC, PE and PI were eluted with 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, by vol.) at a flow rate of 2.0 ml/min. PS was eluted with 30 mM choline chloride in methanol/25 mM KH₂PO₄/acetonitrile/acetic acid (90.5:7:2.5:0.8, by vol.) also at 2.0 ml/min. PC and PE were applied to the column in 50–100 µl of ethanol and PI and PS in 10–20 µl of chloroform. The recoveries of PI, PE and PC were 93.5 % to 103 %, whereas PS recovery was consistently under 90 % (78.5–88.8 %). Likewise, the coefficient of variation of intra- and inter-assays was below 10 % for PE, PC and PI, but 16.4 % for PS. As PS is a minor phospholipid with not much variation with EPO addition, and as PS recovery and coefficient of variation are poorer for the minimal precision set for other phospholipids, we decided not to record the PS molecular species values. Within any class of phospholipid, the order of elution of molecular species was constant and entirely dependent on the composition of the component fatty acid. That is, the RRT of any particular molecular species was the same in all of the phospholipid classes studied. Using the RRT, we developed a graphic relationship that demonstrates that molecular species were eluted in a predictable sequence. Thus, it was possible to predict the effective carbon number in *sn*-1, as well as the position of the unsaturated fatty acid in *sn*-2, for a large number of molecules, which have not actually been identified in measurable amounts in this paper. HPLC analysis of different amounts (1–50 mg) of 16:0,22:6 molecular species gave a linear standard curve, and recovery of 16:0,22:6 DAG from the TLC plates was 102 % and after derivatization was 86 %. The quantification of the molecular species of PC, PE and PI was done directly online from the HPLC detector response in UV at 205 nm and by collecting each peak for GC analysis [19]. Briefly, each peak from the HPLC columns was collected, dried, treated with BF₃/methanol, and analysed by GC. The reason for comparison is that the HPLC method is based on absorbance of double bonds at 205 nm, where completely saturated species (except 16:0,16:0) are not detected. By GC, we also could not detect any 16:0,18:0, 18:0,18:0, or 16:0,16:0 species, confirming that completely saturated species are present at very low levels or are non-existent in the B6SUt.EP cell line. Calibration of most molecular species of PC, PE and DAG was performed in order to compensate for absorbance differences of more saturated versus polyunsaturated species at 205 nm.

Analysis of DAG fatty acid chain linkage

Lipids that contain 1-ether-linked or 1-alkenyl ether-linked fatty acid chains may be eluted differently than ester-linked lipids when otherwise identical lipids are separated by HPLC or by TLC. To demonstrate the chemical linkage between fatty moieties and glycerol in EPO-treated lipids, DAG was generated as described above and converted into ³²P-labelled PA using [³²P]ATP and *Escherichia coli* DAG kinase. It is important to note that *E. coli* DAG kinase phosphorylates 1-palmitoyl-2-acetylgllycerol (1-alkyl) and plasmalogen DAG, generated from plasmalogen PC (1-alkenyl) under these conditions. The resulting

radiolabelled PA was chromatographed on glass-backed silica gel 60 TLC plates. The region of the TLC plates containing the radiolabelled PA, identified by autoradiography, was scraped, extracted [22] and dried under a stream of N₂. The position 1 fatty acid chain linkage of the resulting PA was determined essentially as described by Kennerly [23]. The [³²P]PA obtained as described above was treated with acid with or without subsequent treatment with base due to the fact that alkenyl ether linkages are acid labile and ether linkages are base stable. Acid hydrolysis was performed by treating the PA with 1.0 ml of 0.012 M HCl in methanol and 0.5 ml of chloroform for 15 min at room temperature, extracting the hydrolysis products as described by Bligh and Dyer [22], and drying the organic phase under a stream of N₂. For base hydrolysis, this dried organic extract was treated with 1 ml of 0.45 M NaOH in methanol and 0.5 ml of chloroform for 15 min at room temperature, the hydrolysis products were extracted as described for acid hydrolysis, and the organic phase dried under a stream of N₂. The above organic phases were chromatographed using the TLC system described by Wright et al. [24] to separate the intact PA (*R_F* = 0.74) from any lyso-PA (*R_F* = 0.35) formed during hydrolysis. The regions of the TLC plates containing PA and lyso-PA were scraped and quantified by liquid scintillation counting, as was the radioactivity remaining in the aqueous phases of the above extractions.

Analysis of water-soluble head groups

PC and PE were labelled by incubating cultures in medium which contained 5 mCi/ml [³H]choline and [³H]ethanolamine respectively. We did not label PS. The equilibration with labelled PC and PE was for at least 1 h. Medium was removed, incubations were terminated as described above, and the lipids and water-soluble aqueous phase were dried in a Speed Vac concentrator (Savant). This dried aqueous phase was resuspended in 50 % (v/v) methanol, and an aliquot developed with 0.5 % NaCl/methanol/NH₃ (100:100:2, by vol.) as described [12]. The region of the plate containing the head group and metabolites was scraped and quantified by liquid scintillation counting [25].

RESULTS

Initial experiments were designed to determine the nature of the DAGs stimulated by EPO. Fatty acid chains at position 1 of 1,2-DAG are joined to the glycerol backbone via an ester or by an alkenyl or alkyl ether linkage. The presence of a 1-alkenyl-2-acyl glycerol phosphate would have been indicated by the generation of lyso-PA following acid hydrolysis. Similarly, the generation of lyso-PA following both acid and base hydrolysis would have indicated the presence of a 1-alkyl-2-acyl glycerol phosphate. Taking advantage of the differential stability of the three fatty acid chain linkages in acid and base after conversion of DAG into radiolabelled PA, we could determine the PA and water-soluble components. Alkenyl PA changed from 10 % to 20 % of total PA after 40 min EPO treatment, indicating that this class of PA is being actively generated in preference to diacyl species at longer times of EPO treatment, thus potentially overestimating some diacyl species by 10 %. The amount of lyso-PA formed by acid and base hydrolysis is negligible. Thus, most of the DAG species are diester compounds, and an initial separation to resolve ether lipids from diester compounds was not pursued, although separation of molecular species of alkenyl PC may prove to give additional information on the role of ether lipids in the signal transduction events related to proliferation of these cells.

Figure 1 Temporal patterns of DAG elevation

Data from the distribution of fatty acid molecular species before and after EPO addition were plotted to illustrate the changes in the levels of the different DAG molecular species with regard to time of EPO addition. **(A)** Molecular species whose levels peaked at 10 s after EPO and thereafter returned to control levels. **(B)** Molecular species whose levels remained at the control levels for 10 s or continued to rise and peaked at 40 min. Each point represents the mean from four experiments. The S.D. for different molecular species as a percentage of the total DAG was 0.2–1.2%. Numbers preceded by ‘P’ refer to peak numbers listed in Table 1.

Table 1 shows the molecular species present in cultures without EPO for both DAG and phospholipids (PC, PI, PE and PA). Sixteen different molecular species of DAG were present in significant amounts with peaks 10 (16:0,20:4), 11 (16:0,18:2), 20 (18:0,20:4) and 21 (18:0,18:2) the most abundant species, followed by peak 18 (18:1,18:1). PC had 21 significant molecular species with peaks 10, 11, 20 and 21 as most abundant, followed by peak 18 (18:1,18:1). Interestingly, arachidonate was present in high amounts in the most abundant species. PI had a very different profile of molecular species; although there are 13 significant molecular species, only one (peak 20) is abundant, and there is one moderately abundant peak (26) containing arachidonate (18:1,20:4). PE had 14 significant molecular species with peak 20 (18:0,20:4) as the predominant one (although less of this molecular species was present than in PI), near equal

Figure 2 Comparison of the molecular species profiles of cellular DAG with the profiles of PI, PC and PE

(A) PI-DAG, **(B)** PC-DAG, **(C)** PE-DAG. Profiles are shown at control levels, 10 s after addition of EPO, 40 min after addition of EPO, and 1 h after addition of EPO. The molecular species profile of cellular DAG was compared with the molecular species profiles of corresponding cellular PI, PC and PE by calculating for each molecular species its contribution to the PI, PC or PE profile minus its contribution to the DAG profile. Each number on the abscissa represents a different molecular species (peak) identified in Table 1.

quantities of peaks 9, 10, 11 and 19, and a little less of peaks 12 and 21. PA does not contain species 1–7 and has only 15 significant species with two abundant ones (peaks 19 and 20 with

Table 2 Changes in molecular species of phospholipids 40 min and 1 h after EPO addition

Only decreases of more than 5% were considered significant and are recorded. Each peak represents a molecular species identified in Table 1.

Time after EPO addition	PC		PE		PI	
	Peak no.	Decrease (%)	Peak no.	Decrease (%)	Peak no.	Decrease (%)
40 min	5	6.9	9	7.4	20	11.4
			11	9.1	24	8.3
					25	7.3
					26	5.8
					27	7.2
1 h	5	7.5	9	7.0	20	11.6
			10	8.9	24	8.7
			11	9.2	25	7.2
			12	5.6	26	5.1
			19	6.0	27	7.0

Table 3 Contribution of molecular species of PC and PI to PA

Only molecular species significantly increased in PA were used for calculations (see the Results section). A mean of differences in these molecular species was calculated. For each of these molecular species, its percentage of the phospholipid population was subtracted from its percentage of the PA population with EPO additions at specific times.

	Mean (%)		
	10 s	40 min	1 h
PA—PI	3.4	4.2	4.4
PA—PC	3.1	2.7	2.5

arachidonic acid and docosahexanoic acid) and only somewhat less of peaks 21, 22 and 26.

In view of the kinetics of the stimulation of 1,2-DAG in response to EPO [1], we also examined the effects of EPO on DAG levels after 10 s, 40 min and 1 h of treatment to determine whether increases in molecular species of DAG corresponded to the increase in DAG mass (Figure 1). Only changes in the quantity and not in the presence or absence of specific molecular species were noted. The most abundant fatty acid molecular species (peak 20, containing 18:0 and 20:4) increased after 10 s and continued to increase up to 40 min, then decreased at 1 h, confirming the kinetics shown with increased DAG mass. Some peaks increased only somewhat at 10 s (e.g. peak 11), some only at 40 min (e.g. peak 10), and some did not change significantly. Two important patterns were observed: (1) molecular species that peaked at 10 s (although the changes at 10 s were small, they were more than twice the S.D. above the control and thus significant) and then decreased continuously afterward (peaks 11 and 20; Figure 1A); and (2) other molecular species that increased from 10 s to 40 min (peaks 10, 18 and 19) and decreased thereafter (Figure 1B). These two patterns correspond to the rapid, transient increase in DAG mass and to the slow, sustained (40 min) kinetics shown in our companion paper [1].

Since the molecular species of phospholipids may change in response to EPO in parallel with the DAG species, we determined the effect of EPO at 10 s, 40 min and 1 h on each phospholipid (Table 2). Few changes following EPO addition occurred at 10 s and they are, therefore, not shown, whereas changes in most species at 40 min or 1 h were noted. These decreases are due to hydrolysis of the phospholipids to produce DAG and PA; remodelling of phospholipids takes a longer time than used in our experiments. We calculated that a minimal statistical decrease is more than 5% of the control. We divided these

decreases into two groups, one at 40 min corresponding to the fast increase in DAG at 10 s, and one at 1 h after EPO addition corresponding with the slow increase in DAG. In both cases, the hydrolysis of phospholipids took place later than the corresponding increase in DAG. In the case of PI, a decrease in peak 20 (the most abundant) was observed at 40 min (11.4%) and at 1 h (11.6%). PC demonstrated the most significant decrease at 1 h in peaks 5, 10, 18 and 21 (mean decrease of 7.0%). Interestingly, there was a significant decrease in PE molecular species (peaks 9 and 11) at both 40 min and 1 h (Table 2).

In Figure 2 the comparison of the molecular species of DAG with PI, PC and PE at time 0, 10 s, 40 min and 1 h after EPO addition is shown. These were done by subtracting the molecular species of DAG from the phospholipid population. If all the DAGs at a given time point were derived from the hydrolysis of a single phospholipid, then the difference of the DAG profile from the phospholipid profile should be 0% for each molecular species. Some of the phospholipid peaks were significantly different from DAG. In the case of DAGs after a 10 s treatment with EPO, PE and PI were good matches with the average differences being 2.9 and 3.1%, while the average difference of PC-DAG is 3.9%. At 40 min and 1 h after EPO addition, the PC and PE averages were good matches for DAG (2.0% and 2.3% respectively), whereas PI (3.8%) was not a good match (Figure 2).

The same type of calculations were done for a comparison of molecular species of PA with PI and PC at different times of EPO treatment (Table 3). Only the molecular species increases in PA were used for comparison (18:0,22:6; 18:0,20:4; 18:0,18:2; 18:0,18:1 and 18:1,20:4). A mean of differences was calculated at 10 s, 40 min and 1 h in order to assess the contribution of EPO addition to the hydrolysis of PI and PC and the fast increase in PA, as previously observed by us [1]. If all of the PA at a given time point was derived from the hydrolysis of a single phospholipid (PI or PC), then the differences of the PA profile from that of PI or PC should again be 0% for each molecular species. Both PC and PI molecular species were different from PA, but by calculating the mean of all differences, PC was a better match than PI.

Because of the unexpected similarity of the molecular species profiles of PE at all times and PC at fast times to DAG, it was important to examine the release of phospholipid head groups following EPO treatment. Table 4 illustrates the release of choline and ethanolamine head groups from control cells and EPO-treated ones. At 20 s, 40 min and 1 h there was a significant release of ethanolamine in response to EPO ($P < 0.005$ at 20 s; $P < 0.05$ at 40 min and 1 h), implicating PE as a source of DAG in fast and slower DAG kinetics profiles. In addition, choline was released in significant amounts at 40 min and at 1 h ($P < 0.05$), implicating PC for the slower DAG release. However, we should

Table 4 Release of choline and ethanolamine head groups from control cultures and after EPO addition in cultures of B6Sut.EP cells

B6Sut.EP cell cultures were labelled with [^3H]choline or [^3H]ethanolamine. EPO was added for 20 s, 40 min, or 1 h. Incubations were terminated, water-soluble components extracted, and head group metabolites separated by TLC. The region of the plate containing the metabolites was scraped and quantified by liquid scintillation counting as described in the Materials and methods section. In parentheses, data are expressed as percentages of control levels (mean $\pm$ S.E.M.). * $P < 0.05$; ** $P < 0.005$.

	Radioactivity released (c.p.m./mg of protein)			
	Control	EPO 20 s	EPO 40 min	EPO 1 h
Choline	3088 $\pm$ 148	2737 $\pm$ 186	3498 $\pm$ 180 (113 $\pm$ 3)*	3626 $\pm$ 136 (117 $\pm$ 4)*
Ethanolamine	6845 $\pm$ 338	9589 $\pm$ 520 (140 $\pm$ 6)**	8956 $\pm$ 400 (131 $\pm$ 7)*	8624 $\pm$ 344 (126 $\pm$ 5)*

note that, although ethanolamine is converted into choline in many cell types, it has not been determined if this occurs in B6Sut.EP cells.

DISCUSSION

Treatment of cultured B6Sut.EP cells with EPO resulted in stimulation of both DAG and PA lipid signalling molecules with a characteristic biphasic response to EPO [1]. In this paper, we have confirmed that the increase in cellular DAG levels corresponded to increases in specific molecular species. We also noted two patterns: (1) an increase of 16:0,18:2 and 18:0,20:4, which parallels the rapid and transient increase in DAG mass; and (2) a slow and sustained increase in molecular species 16:0,20:4, 18:1,18:1 and 18:0,22:6, which parallels the second phase of DAG production. Using sensitive HPLC methods for direct identification of individual molecular species of PC, PE and PI, we found variable decreases in specific molecular species of these phospholipids due to hydrolysis [i.e. some decreases occurring faster (in PE, PI and possibly PC) and some slower (in PE and PC)].

In light of the evidence implicating these phospholipids as the major sources of EPO-stimulated DAG, we compared the molecular species of PI, PC and PE with those of DAG. In order to compare the profile of EPO-stimulated DAG with those of cellular phospholipids, we subtracted each phospholipid molecular species at each time after EPO addition from the corresponding DAG molecular species at the same times. For any 'difference' profile to approach zero, both the EPO-stimulated and resting DAG would have to be derived from the phospholipid. According to our findings, none of the profiles approached zero, supporting the conclusion that no single phospholipid can be hydrolysed to produce all DAG species observed. However, it appears from Figure 2 that PC can produce the right amounts of all species to match the DAG profile except for species 20. Since species 20 is the major component of PI, a combination of PC and PI hydrolysis would be sufficient to account for the DAG species seen. A mean of the differences of all molecular species gave better and more precise information; some hydrolysed phospholipids showed a better mean fit than others at specific times after EPO addition. By these calculations, important molecular species profiles of PI resembled that of DAG only at 10 s. Molecular species of PC were a better fit to that of DAG at 40 min and 1 h. Unexpectedly, the best fit (mean closer to 0) was for PE, both at short and longer EPO treatment times. This finding was confirmed by the release of ethanolamine-containing head groups at all times, whereas release of choline-containing head groups was only significant at longer EPO times. Therefore, while PI contributes only a small proportion of DAG at 10 s, PC contributes significantly at 10 s and 40 min, and PE is also a major contributor to DAG generated after 10 s and 1 h of EPO addition.

Our study and reports by others [23,25] indicate that only a small percentage of the stimulated DAG arises from hydrolysis of PI. As for many other agonists, there are major quantitative discrepancies between the phosphatidylinositol bisphosphate present or disappearing and the DAG accumulating [24–28]. Of greater importance is the observation that EPO produced prolonged hydrolysis of PE. The mechanisms by which EPO or other growth factors promote PE breakdown are unknown but may involve tyrosine phosphorylation of relevant phospholipases (A_2 , C and D) and/or additional proteins. Therefore, it is reasonable to propose that the products of PE, as well as PC hydrolysis, may serve as second messengers for signal transduction of cellular responses, and that PE breakdown is involved

in control mechanisms that require prolonged activation of PKC [5]. In cells exhibiting the transformed phenotype and in certain human tumours, PC and PE are elevated, consistent with the increased breakdown of both PE and PC [12,13].

The production of large amounts of certain species of DAG (16:0,20:4 and 18:0,20:4) in response to EPO may be the result of either an EPO-stimulated phospholipase C having specificity for phospholipids containing these species or of a predominance of these phospholipids at the intracellular location(s) where this phospholipase is activated. One other explanation would also fit these results. The phospholipase C could be highly specific for lipids with palmitate and stearate at the *sn*-1 position and a C_{20} polyunsaturated fatty acid at the *sn*-2 position. Similar changes were observed in Swiss 3T3 fibroblasts on epidermal growth factor stimulation [29]. One other possibility is that cells contain a DAG kinase activity selective for C_{20} species, which is inhibited by tyrosine phosphorylation. On the other hand, the more general increase in other DAG species could result from the involvement of a different phospholipase C(s), or it could be due to an inhibitory action of EPO on DAG lipase [30]. If we assume that the primary function of EPO-stimulated DAG is to activate PKC through phospholipases as implicated by our earlier studies [2,3] and those of others [4], then specific PKC isoforms may be differentially sensitive to specific molecular species of DAG (e.g. those containing arachidonic or docosahexaenoic acid) [31]. However, DAG has other functions independent of PKC. One is to serve as a source of arachidonic acid for eicosanoid production. In this study hydrolysis of all three principal phospholipids with EPO stimulation increased the hydrolysis of molecular species which contained 20:4 (PC and PE, peak 10; PI, peak 20) or 20:6 (PE, peaks 9 and 19), which also are involved in the generation of prostaglandins and leukotrienes. It is possible that multiple pools of DAG exist in different subcellular sites, each with potentially different pathways of DAG formation and subsequent metabolism as described for mast cells [23]. The possibility that an EPO-activated phospholipase exists with a preference for a subpopulation of a phospholipid with a particular acyl structure should be considered [32].

Since PA is formed much more rapidly than DAG in response to EPO and may accumulate to a level comparable with DAG, it is possible that it is generated mainly in the plasma membrane where phospholipase D resides, as well as G-proteins and EPO receptors. In experiments with HL-60 granulocytes incubated with a double label and alkyl-lyso-PC, Billah and his co-workers [33] showed that the early appearance of alkyl PA is due to phospholipase D and not phospholipase C. Our results (Table 4) show convincingly that changes in molecular species of PA with EPO involve PC primarily but not PI. Therefore, PI cannot account for the mass of PA formed during the fast phase after incubation with EPO nor for the PA formed during 30 min with EPO. Our earlier studies with murine fetal liver cells also found no evidence for phosphatidylinositol bisphosphate hydrolysis in response to EPO [2]. It is likely that phospholipases C and D, as well as phospholipase A_2 [2], are involved in the signal transduction events triggered by EPO. PKC, which has been implied to be essential for the proliferative effects of EPO [2–4], would then be continually activated by PC hydrolysis, as well as PE hydrolysis.

The physiological roles of the large amount of PA generated remain unknown but may involve stimulation of Ca^{2+} influx and/or stimulation of arachidonic acid release, DNA synthesis, inhibition of GAP activity [7], or activation of PKC [34]. Phospholipase D has been shown to be both activated by PKC in fibroblasts [35] and inhibited by PKC in formylmethionyl-leucyl-phenylalanine-stimulated rabbit peritoneal neutrophils

[36]. Overexpression of PKC β in fibroblasts greatly enhanced phospholipase D activation by phorbol 12-myristate 13-acetate, and the activation was inhibited by staurosporine, a PKC inhibitor [37].

In conclusion, the present findings provide further support for the concept that EPO induces the hydrolysis of PC and PE in target cells. Hydrolysis of these phospholipids provides a means of generating large concentrations of DAG and PA. In the later phases of EPO stimulation when PI hydrolysis is diminished, cellular responses may depend on continued activation of PKC via PC and PE. The potential sources of PA identified by incubating B6Sut.EP permeabilized cells with guanosine 5'-[γ -thio]triphosphate [1] were PC as a major source, with PE as an additional one. Our results indicate that although the stimulation of B6Sut.EP cells by EPO resulted in the hydrolysis of PI in the early phase, the majority of DAGs and PA are generated from PC and PE hydrolysis. The findings are consistent with a growing number of studies demonstrating the importance of PC and PE in prolonged biological responses that require lipid signalling molecules.

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