

# Selective decrease of bis(monoacylglycero)phosphate content in macrophages by high supplementation with docosahexaenoic acid<sup>S</sup>

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**Abstract** Bis(monoacylglycero)phosphate (BMP) is a unique phospholipid (PL) preferentially found in late endosomal membranes, where it forms specialized lipid domains. Recently, using cultured macrophages treated with anti-BMP antibody, we showed that BMP-rich domains are involved in cholesterol homeostasis. We had previously stressed the high propensity of BMP to accumulate docosahexaenoic acid (DHA), compared with other PUFAs. Because phosphatidylglycerol (PG) was reported as a precursor for BMP synthesis in RAW macrophages, we examined the effects of PG supplementation on both FA composition and amount of BMP in this cell line. Supplementation with dioleoyl-PG (18:1/18:1-PG) induced BMP accumulation, together with an increase of oleate proportion. Supplementation with high concentrations of didocosahexaenoyl-PG (22:6/22:6-PG) led to a marked enrichment of DHA in BMP, resulting in the formation of diDHA molecular species. However, the amount of BMP was selectively decreased. Similar effects were observed after supplementation with high concentrations of nonesterified DHA. Addition of vitamin E prevented the decrease of BMP and further increased its DHA content. Supplementation with 22:6/22:6-PG promoted BMP accumulation with an enhanced proportion of 22:6/22:6-BMP. DHA-rich BMP was significantly degraded after cell exposure to oxidant conditions, in contrast to oleic acid-rich BMP, which was not affected. Using a cell-free system, we showed that 22:6/22:6-BMP is highly oxidizable and partially protects cholesterol oxidation, compared with 18:1/18:1-BMP. Our data suggest that high DHA content in BMP led to specific degradation of this PL, possibly through the diDHA molecular species, which is very prone to peroxidation and, as such, a potential antioxidant in its immediate vicinity.—Bouvier, J., K. A. Zemski Berry, F. Hullin-Matsuda, A. Makino, S. Michaud, A. Geloën, R. C. Murphy, T. Kobayashi, M. Lagarde, and I. Delton-Vandenbroucke.

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Bis(monoacylglycero)phosphate (BMP), also known as lysobisphosphatidic acid, is a unique phospholipid (PL) highly enriched in the internal membranes of multivesicular endosomes, in which it forms specialized lipid domains (1–3). BMP is involved in the formation of the multivesicular membrane organization of late endosomes (4, 5). It has been suggested that BMP participates in the trafficking of lipids and proteins, particularly cholesterol, through late endosomes (6–8). Recent studies have indicated that the ability of the endosomal Niemann-Pick C2 protein to transfer cholesterol to PL vesicles is increased by the presence of BMP (9, 10). This PL acts as a cofactor of sphingolipid degradative enzymes in late endosomes (11). It has been reported to enhance the activity of lysosomal acid lipase *in vitro* (12). It plays an important role in the fusion event necessary for the cytoplasmic release of the vesicular stomatitis virus (7). BMP has also been associated with anti-PL syndrome because it is a specific antigen of the antibodies found in these patients (1, 13, 14).

Abbreviations: BHK, baby hamster kidney; BHT, butylated hydroxytoluene; BMP, bis(monoacylglycero)phosphate; CL, cardiolipin; DHA, docosahexaenoic acid; DMA, dimethyl acetal; FAME, fatty acid methyl ester; MDA, malondialdehyde; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid.

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BMP is a structural isomer of phosphatidylglycerol (PG). Whereas PG binds two acyl groups to one glycerol moiety, BMP binds one acyl group to each of the two glycerol moieties. In addition, BMP has an unusual *sn*-1-glycerophospho-*sn*-1'-glycerol configuration (15, 16). In most cell types, the FA composition of BMP is characterized by a high proportion of oleic acid (OA). With respect to PUFAs, BMP was found to be rich in the *n*-3 FA docosahexaenoic acid (DHA) in several cells and organs, including uterine stromal U<sub>III</sub> cells (17), PC12 cells (18), THP-1 macrophages (19), and rat liver (20). We and others have previously stressed the high propensity of BMP to incorporate and accumulate exogenous DHA, even in cells such as baby hamster kidney (BHK) cells, whose BMP contains low endogenous amounts of DHA (17, 19, 21). Indeed, in both THP-1 macrophages and BHK cells, we showed that DHA was selectively incorporated into BMP, compared with other PUFAs such as arachidonic acid. In addition, DHA was more efficiently enriched in BMP compared with other PLs such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). DHA has interesting medical applications, inasmuch as its dietary uptake has been positively linked to the prevention of inflammatory and cardiovascular diseases (22). The content of DHA in biological membranes has been shown to influence membrane properties such as fluidity, fusion, and raft formation (23, 24). Of note, it has been reported that membranes enriched with DHA-containing PLs exclude cholesterol (24). We have been particularly interested in the role of BMP in the regulation of cholesterol homeostasis in macrophages (8), and DHA content of BMP might be important in this respect.

In this context, it is important to clarify both the mechanisms and the biological significance of DHA enrichment in BMP. Numerous studies have clearly established that exogenous PG and cardiolipin (CL) could serve as precursors for BMP synthesis (25–28). Recently, we have reported that PG, but not CL, is an endogenous precursor of BMP (29). Of interest, Waite et al. (27) have pointed out some variability in the level of BMP synthesis with PG derived from different PL precursors, which they proposed to be related to differences in PG FA composition. Here, we further examined whether the nature of PG could influence BMP synthesis and its FA composition in RAW macrophages. In addition to the biochemical aspect, we were also interested in setting up experimental conditions that would induce specific quantitative (mass) and/or qualitative (DHA content) changes of BMP in the perspective of functional studies.

Our results show that supplementation of RAW macrophages with dioleoyl-PG (18:1/18:1-PG) resulted in a significant BMP accumulation in these cells, with a parallel increase of OA proportion in BMP. Supplementation with didocosahexaenoyl-PG (22:6/22:6-PG) promoted a high enrichment of DHA in BMP. This indicates that FA composition of BMP varies according to the PG precursor. However, we found that the amount of BMP was selectively decreased in the presence of high concentrations of 22:6/22:6-PG. This unexpected observation led us to focus on the mechanism of BMP decrease in relation to DHA incor-

poration. Our findings suggest that the high capacity of BMP to incorporate DHA may expose it to subsequent degradation, possibly through the formation of the diDHA molecular species, which is very prone to peroxidation. Such a high sensitivity could make 22:6/22:6-BMP a potential antioxidant in its immediate vicinity.

## EXPERIMENTAL PROCEDURES

### Materials

Tissue culture medium, reagents, and serum were purchased from Eurobio (Les Ulis, France). *Cis*-4,7,10,13,16,19-docosahexaenoic acid (22:6n-3), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine (17:0/17:0-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0/16:0-PC), 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (18:1/18:1-PG), and vitamin E were purchased from Sigma (Saint Quentin Fallavier, France). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (17:0/17:0-PC), *sn*-(3-oleoyl-2-hydroxy)-glycero-11-phospho-*sn*-3'-(1'-oleoyl-2'-hydroxy)-glycerol (18:1/18:1-BMP), and 1,2-didocosahexaenoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (22:6/22:6-PG) were from Avanti Polar Lipids (Alabaster, AL). 1,2,3-Tridocosahexaenoyl-*sn*-glycerol (tri22:6-TG) was from Polarix (Pleuven, France). *sn*-(3-Pentadecanoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1'-(3'-pentadecanoyl-2'-hydroxy)-glycerol (15:0/15:0-BMP) and *sn*-(3-docosahexaenoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1'-(3'-docosahexaenoyl-2'-hydroxy)-glycerol (22:6/22:6-BMP) were chemically synthesized by our chemist colleagues according to the method previously described (30). [ $1\alpha,2\alpha$ -( $n$ - $^3$ H)]cholesterol was from GE Healthcare (Saclay, France). All solvents were of analytical grade from SDS (Peypin, France). Silica gel 60 plates were supplied by Merck (Fontenay Sous Bois, France).

### Preparation of PG liposomes

PG liposomes were prepared extemporaneously by transferring 18:1/18:1-PG or 22:6/22:6-PG in chloroform to a glass tube and evaporating the solvent in a vacuum for 30 min. The lipid residue was then dispersed in 200  $\mu$ l of PBS solution (5 mM final) by vortexing for 15 min at 4°C. The multivesicular liposomes were then sonicated for 15 min at 4°C to make small liposomes. Quantification of FAs by GC analysis in the resulting liposome suspensions indicated no degradation of initially added PG. PG liposomes, including vitamin E, were prepared using the same procedure after addition of vitamin E in ethanol solution (1 molecule for 100 unsaturations) at the initial step.

### Cell culture and treatment

Murine macrophage-like RAW 264.7 cells were obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were maintained in MEM supplemented with non-essential amino acids, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (control medium). They were routinely grown in 100 mm dishes at 37°C in an atmosphere of 5% CO<sub>2</sub> and subcultured by trypsinization at a 1:3 ratio. When indicated, culture medium was supplemented with 5  $\mu$ M vitamin E. An adequate volume of ethanolic solution of vitamin E was dried under nitrogen, and the residue was suspended in serum, vortexed for 5 min, and maintained overnight under gentle agitation at 37°C, before addition to the medium. For experiments, RAW macrophages were seeded into 100-mm-diameter dishes at a density of  $7 \times 10^6$  cells per dish. After 24 h, the medium was removed and replaced by control medium or medium supplemented with PG liposomes or nonesterified DHA. The different PG liposome suspensions were added into the culture medium at final concen-

trations of 5  $\mu\text{M}$  to 60  $\mu\text{M}$ . For supplemented with nonesterified DHA, the highest concentration was fixed to 100  $\mu\text{M}$  with a molar ratio to serum albumin  $\leq 2$  to avoid deleterious effects of excess FFA. An adequate volume of ethanolic solution of DHA (final 100  $\mu\text{M}$  in medium) with or without vitamin E (1 molecule for 100 unsaturations) was dried under nitrogen, and the residue was suspended in serum, vortexed for 5 min, and maintained overnight under gentle agitation at 37°C, before addition to the medium. Media supplemented with 60, 30, 15, and 5  $\mu\text{M}$  DHA were obtained by successive dilutions. Cells were incubated with nonesterified DHA or PG liposomes for 24 h. In some experiments, cells were subsequently exposed to oxidant conditions (1 mM  $\text{H}_2\text{O}_2$ , 0.1 mM  $\text{CuSO}_4$ , 1 mM ascorbate, 4 h) in the absence of vitamin E. At the end of incubation, cells were rinsed three times with PBS, scraped in PBS, and pelleted by centrifugation. Cell pellets were kept frozen at  $-20^\circ\text{C}$  until analysis.

### PL analysis

Cell pellets from four dishes exposed to the same treatment were pooled. Cell lysates (0.1% Triton in water) were analyzed for protein content using the Bradford assay. Total lipids were extracted by the method of Bligh and Dyer (31) after addition of butylated hydroxytoluene (BHT, 50  $\mu\text{M}$  final) as an antioxidant and internal standards (50  $\mu\text{g}$  17:0/17:0-PC, 25  $\mu\text{g}$  17:0/17:0-PE, and 4  $\mu\text{g}$  15:0/15:0-BMP). Lipids were first separated by two-dimensional (2D)-TLC (silica gel 60 plates 20  $\times$  20 cm). The first dimension was run with chloroform-methanol-32% ammonia solution (65:35:5, by volume) and the second dimension with chloroform-acetone-methanol-acetic acid-water (50:20:10:12.5:5, by volume). Lipids were detected by ultraviolet light after spraying with 0.05% primulin in methanol and identified by comparison with standards. Using this 2D-TLC system, PC is well-separated from other lipids, whereas PE comigrates with PG and BMP migrates very close to CL. The silica gel areas containing PC were scraped and transferred to a glass tube. The silica gel areas containing BMP (and possibly CL) and PE/PG were scraped, and lipids were extracted using a chloroform-methanol-water mixture. Purification of BMP and PE was carried out on a Nucleosil  $\text{NH}_2$  column (5  $\mu\text{m}$ , 250  $\times$  4.6 mm) using a Hewlett-Packard 1100 HPLC system equipped with a quaternary pump and a diode array detector as previously described (17). The PLs (PC, PE, BMP) were transmethylated using 5%  $\text{H}_2\text{SO}_4$  in methanol. The resulting fatty acid methyl esters (FAMES) and fatty aldehyde dimethyl acetals (DMAs) were analyzed by GC using a Hewlett-Packard system equipped with a Supelco SP2380 capillary column (60 m  $\times$  0.22 mm), with helium as a carrier gas. FAMES and DMAs were identified by comparison with commercial standards (17). Results are expressed as mole percent (mol%) of each individual FA calculated from the sum of all detected FAs taken as 100%. Quantification of PL was made in correspondence with their FA content using the internal standards.

### Determination of BMP molecular species by electrospray MS

BMP was purified from RAW cells, as described previously, by 2D-TLC and HPLC. The molecular species composition was analyzed by electrospray MS. The BMP molecular species were infused using a NanoMate 100 (Advion BioSciences, Ithaca, NY) into a Sciex API 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (PE Sciex, Toronto, Canada). The concentration of the sample that were infused in these experiments was approximately 100 nM in methanol-dichloromethane (1:1, by volume). Nanoelectrospray was initiated using the NanoMate by applying a  $-1.32$  kV spray voltage and a 0.15 psi nitrogen head pressure to the sample in the pipette tip. The typical flow rates achieved using these settings were 200 nl/min. For full scan and enhanced product ion experiments in the negative-

ion mode, a declustering potential of  $-80$  V was used. Additionally, for the enhanced product ion scans, the collisional offset was 40 V and the collisionally activated dissociation gas was set at high. The mole percents reported are based on the molecular ion abundance of BMP molecular species after correction for naturally occurring isotopes (32).

### Malondialdehyde determination

Malondialdehyde (MDA) was measured by reverse-phase (RP)-HPLC of the MDA-thiobarbituric acid (TBA) adduct according to the method of Therasse and Lemonnier (33). Briefly, cells were treated with 10 mM TBA dissolved in 0.1 M phosphate buffer, pH 3.0, and incubated for 1 h at 95°C in the presence of acetic acid and BHT. The MDA-TBA adduct was then extracted with ethylacetate and separated by HPLC on a 25 cm column packed with Nucleosil C-18 (5  $\mu\text{m}$ ). The mobile phase water-methanol (80:20, by volume) was eluted at a flow rate of 0.8 ml/min. The MDA-TBA adduct was measured by fluorometry ( $\lambda$  excitation = 515 nm,  $\lambda$  emission = 553 nm). The standard curve was obtained using 1,1,3,3-tetramethoxypropane as standard (0–100 pmol). Results were expressed as pmol/mg protein, by extrapolation from the standard curve.

### Vitamin E determination

Vitamin E ( $\alpha$ -tocopherol) determination was performed according to a previously described method (34). Briefly, cells were resuspended in 800  $\mu\text{l}$  of ethanol-water (1:1, by volume) containing tocol (10 pmol) as an internal standard for quantification. After two extractions with 1 ml hexane,  $\alpha$ -tocopherol was separated by RP-HPLC on a 15 cm column packed with Nucleosil C-18 (5  $\mu\text{m}$ ) using methanol-water (98:2, by volume) as a mobile phase (1 ml/min) and detected by fluorometry ( $\lambda$  excitation = 295,  $\lambda$  emission = 340). Results were expressed as pmol/mg protein.

### Oxidation of BMP and cholesterol in a cell-free system

Liposomes [16:0/16:0-PC-BMP-cholesterol (75:20:5, mol%)] were prepared by mixing 16:0/16:0-PC, 18:1/18:1-BMP, or 22:6/22:6-BMP, cholesterol and [ $^3\text{H}$ ]cholesterol (0.5  $\mu\text{Ci}$ ) with or without vitamin E (1 molecule for 100 unsaturations). After drying under vacuum, the lipid residue was dispersed in PBS by vortexing for 10 min at 4°C. The resulting liposomes were then exposed to oxidant conditions by incubation with 100  $\mu\text{M}$   $\text{CuSO}_4$  and 10 mM  $\text{H}_2\text{O}_2$  for 2 to 6 h at 40°C. The oxidation was stopped by addition of 50  $\mu\text{M}$  BHT. Lipids were then extracted, and one fraction of the lipid extract was run in 1D-TLC using an acidic system (chloroform-acetone-methanol-acetic acid-water, 50:20:10:12.5:5, by volume) to separate BMP from other lipids. BMP was visualized by spraying with 3% cupric acid-10% phosphoric acid in ethanol and heating at 180°C for 10 min. Another fraction of the lipid extract was run in 1D-TLC using hexane-diethyl ether-methanol-acetic acid (50:50:5:1, by volume) to separate free cholesterol and oxysterols. Lipids were identified by comparison with standards (7-hydroxy-, 25-hydroxy-, and 7-keto-cholesterol). The silica gel areas containing oxysterols and cholesterol were scraped, and the radioactivity was counted by liquid scintillation. Cholesterol oxidation was determined as the proportion of total radioactivity (free and oxidized cholesterol) associated to oxysterols.

### DHA supplementation of mice

Experiments and installations were approved by the French Ministry of Agriculture, Fishing and Alimentation, and the Departmental Veterinary Agency of Rhône. All animals were treated according to guidelines approved by the European Communities Council (November 24, 1986, 86/609/EEC). Three-week-

old male International Cancer Research mice (C57BL/6J, Gannat, France) were fed standard diet A03 (SAFE, Augy, France) containing 5% lipids, or a DHA-enriched diet reconstituted with lipid-free powder (SAFE, Augy, France), 4.5% sunflower oil (Lesieur, Asnières-sur-Seine, France) and 0.5% 1,2,3-tridocosahexaenoyl-glycerol (Polariz, Pleuven, France). Both diets are nutritionally balanced. The complete nutrient and FA composition of diets is available as supplementary material (see supplementary Tables I, II). After 32 days of the respective diet, mice were euthanized by lethal intraperitoneal injection of pentobarbital. The liver was immediately removed, frozen in liquid nitrogen, and kept at  $-80^{\circ}\text{C}$ . Liver (250 mg) was homogenized in 5 ml water using homogenizer, and lipids were extracted by the method of Bligh and Dyer (31). Total lipids were separated by 2D-TLC/HPLC, and FA composition of PC, PE, and BMP was determined as described above.

### Statistical analyses

The results are presented as means  $\pm$  SD of at least three independent experiments or as means  $\pm$  SD of four values from one experiment representing three independent experiments. All statistical analyses were performed using the JMP 5.0.1.2 software (SAS Institute, Inc.). Multiple comparisons were performed by one-way or two-way ANOVA. Means comparisons of all pairs were performed by the Tukey-Kramer Honestly Significant Difference method based on ANOVA with  $\alpha = 0.05$ ; significantly different pairs are indicated in the figures by different capital letters. Experimental means expressed as percent of control were compared with the theoretical control value of 100% using a one-sample Student's *t*-test; differences significant at  $P \leq 0.05$  and  $P \leq 0.01$  are indicated in the figures by \* and \*\*, respectively. Comparisons of experimental means from two groups were performed by two-sample Student's *t*-test.

## RESULTS

### Supplementation with 18:1/18:1-PG liposomes increased BMP content in RAW macrophages

The amount of BMP in RAW macrophages grown under control conditions is  $3.2 \pm 0.9$  nmol/mg protein (mean  $\pm$

SD of five independent determinations), as measured from quantification of FAs using 15:0/15:0-BMP as an internal standard. As in other cell types including THP-1 macrophages, BMP is a minor PL because as it corresponds to about 3% of total PLs, compared with 49% ( $51.3 \pm 6.9$  nmol/mg protein) and 26% ( $27.8 \pm 4.5$  nmol/mg protein) for PC and PE, respectively. The major FA of BMP is OA (18:1n-9), which represents nearly 50% of total FAs (Table 1). BMP is rich in n-3 compared with n-6 polyunsaturated FAs, especially DHA (22:6n-3), which is 10-fold more abundant than arachidonic acid (20:4n-6).

After 24 h incubation with 18:1/18:1-PG, BMP content was significantly augmented in a dose-dependent manner. It was almost double, with as low as  $5 \mu\text{M}$  of 18:1/18:1-PG and augmented up to 4-fold with  $60 \mu\text{M}$  (Fig. 1). Of note, the increase was specific to BMP, inasmuch as the content of both PC and PE was unchanged. Furthermore, there was no accumulation of 18:1/18:1-PG and no change of CL content under these conditions (not shown). The oleate proportion in BMP progressively rose to a maximum close to 75% of total FAs in cells treated with  $30 \mu\text{M}$  18:1/18:1-PG (Table 1). The conversion of percentage values to mass values for each FA in BMP revealed that oleate was selectively increased compared with other FAs, parallel to the increase in the amount of BMP, which is consistent with the quasi-exclusive synthesis of 18:1/18:1-BMP (not shown). This indicates that acylation of BMP was primarily made with the FAs originating from PG.

### Supplementation with 22:6/22:6-PG liposomes conversely decreased BMP content when associated with high incorporation of DHA

Because BMP accumulation was close to saturation at  $30 \mu\text{M}$  18:1/18:1-PG, the same concentration and a lower one were selected for supplementation with 22:6/22:6-PG. At  $10 \mu\text{M}$ , 22:6/22:6-PG promoted only a small increase in the amount of BMP (Fig. 2). Unexpectedly, at  $30 \mu\text{M}$  22:6/

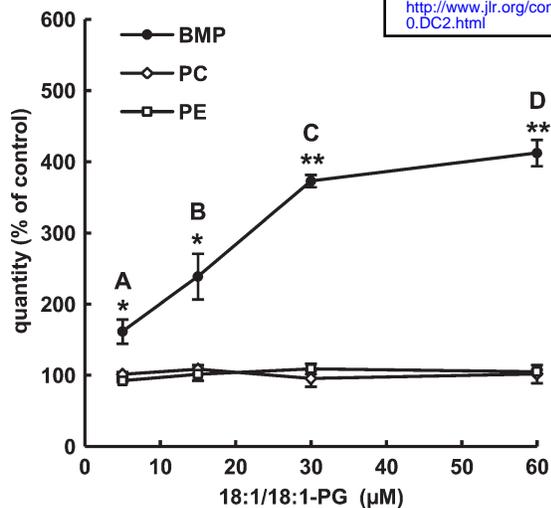
TABLE 1. FA composition of BMP

FA	Control	18:1/18:1-PG (30 $\mu\text{M}$ )	22:6/22:6-PG (30 $\mu\text{M}$ )		DHA (30 $\mu\text{M}$ )	
			-Vitamin E	+Vitamin E	-Vitamin E	+Vitamin E
<i>mol %</i>						
14:0	$0.5 \pm 0.2$	$0.2 \pm 0.1$	$0.9 \pm 0.2$	$0.2 \pm 0.1$	$0.7 \pm 0.3$	$0.5 \pm 0.1$
16:0	$7.7 \pm 1.4$	$2.7 \pm 0.8$	$8.9 \pm 1.4$	$1.7 \pm 0.4$	$8.4 \pm 1.3$	$4.9 \pm 1.2$
18:0	$5.9 \pm 0.8$	$2.6 \pm 0.5$	$6.8 \pm 1.1$	$1.1 \pm 0.3$	$5.5 \pm 0.7$	$4.5 \pm 1.9$
16:1n-9/n-7	$4.4 \pm 0.8$	$2.3 \pm 0.4$	$1.9 \pm 0.5$	$0.7 \pm 0.1$	$1.5 \pm 0.7$	$1.1 \pm 0.1$
18:1n-9	<b><math>51.2 \pm 3.0</math></b>	<b><math>73.8 \pm 0.4^a</math></b>	$23.0 \pm 4.2$	$5.8 \pm 0.8$	$16.7 \pm 2.4$	$11.1 \pm 3.9$
18:1n-7	$16.8 \pm 2.9$	$5.8 \pm 1.7$	$7.4 \pm 2.3$	$1.4 \pm 0.3$	$3.1 \pm 0.4$	$3.3 \pm 1.6$
18:2n-6	$1.2 \pm 0.2$	$1.0 \pm 0.5$	$1.6 \pm 0.8$	$0.5 \pm 0.2$	$1.3 \pm 0.3$	$0.7 \pm 0.1$
20:4n-6	$0.7 \pm 0.1$	$0.7 \pm 0.2$	$0.3 \pm 0.1$	$0.2 \pm 0.1$	$0.4 \pm 0.1$	$0.3 \pm 0.1$
22:5n-3	$2.1 \pm 0.6$	$1.9 \pm 0.9$	$0.9 \pm 0.6$	$1.7 \pm 0.8$	$0.9 \pm 0.3$	$2.0 \pm 1.3$
22:6n-3	<b><math>7.4 \pm 2.7</math></b>	$4.7 \pm 1.7$	<b><math>48.5 \pm 7.1^a</math></b>	<b><math>84.7 \pm 4.7^{a,b}</math></b>	<b><math>59.4 \pm 3.8^a</math></b>	<b><math>71.1 \pm 3.1^{a,b}</math></b>

BMP, bis(monoacylglycerol)phosphate; PG, phosphatidylglycerol; DHA, docosahexaenoic acid. RAW macrophages were cultured for 24 h in the absence or presence of  $30 \mu\text{M}$  18:1/18:1-PG,  $30 \mu\text{M}$  22:6/22:6-PG, or  $30 \mu\text{M}$  DHA, with or without vitamin E for the two later conditions. After total lipid extraction and separation by TLC/HPLC, BMP was analyzed for fatty acid composition by GC as described in Experimental Procedures. Data are expressed as mole percent and represent means  $\pm$  SD from at least three independent determinations. Quantitatively minor FAs (less than 0.5% of total) were included for calculation but not tabulated. Significant differences are only reported for bold values.

<sup>a</sup>  $P \leq 0.05$  versus control (two-sample *t*-test).

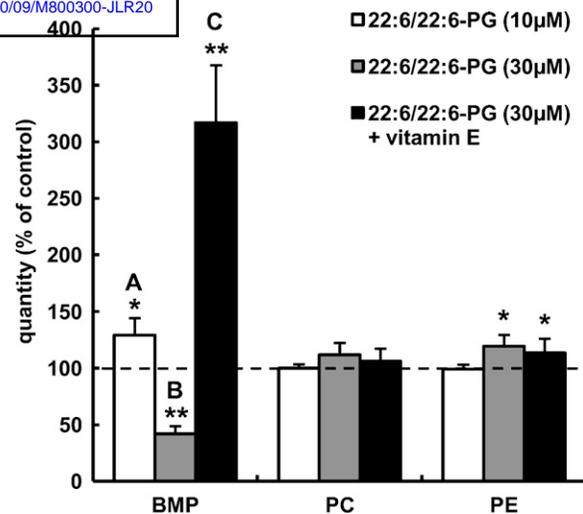
<sup>b</sup>  $P \leq 0.05$  versus the respective condition without vitamin E (two-sample *t*-test).



**Fig. 1.** Variation of bis(monoacylglycerol)phosphate (BMP), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) amounts after supplementation with 18:1/18:1-PG. RAW macrophages were cultured in the absence or presence of increasing concentrations of 18:1/18:1-phosphatidylglycerol (PG) for 24 h. After total lipid extraction and separation by TLC/HPLC, phospholipids (PLs) were analyzed for FA composition by GC and quantified as described in Experimental Procedures. Absolute amounts of PLs were measured in nmol/mg protein and expressed as percentage of control values. Data represent the means  $\pm$  SD from at least three independent determinations. A, B, C, D: significantly different groups (multiple means comparisons by ANOVA and Tukey-Kramer method with  $\alpha = 0.05$ ). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$  versus control (one-sample *t*-test).

22:6-PG, the amount of BMP was significantly decreased compared with controls. Cell viability was not affected under these conditions ( $98 \pm 4\%$  of control cells as assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide test, mean  $\pm$  SD of three independent determinations in five repeated wells), indicating no general cellular toxic effect. In comparison with OA, which contains only one unsaturation, DHA is known to be highly sensitive to autoxidation/peroxidation due to its high degree of unsaturation (35, 36). We have then examined the effect of the antioxidant vitamin E. Of note, the addition of vitamin E to liposomes not only prevented the loss of BMP but triggered instead a 3-fold accumulation of this PL. In contrast to BMP, the quantity of PC was unchanged and that of PE was only slightly increased after incubation with 22:6/22:6-PG in either the absence or the presence of vitamin E (Fig. 2).

Of interest, variations in the amount of BMP were associated with important changes of DHA proportion in this PL (Table 1). The incubation with  $10 \mu\text{M}$  22:6/22:6-PG induced a moderate increase, by 2-fold, of DHA proportion in BMP (not shown). After incubation with  $30 \mu\text{M}$  22:6/22:6-PG in the absence of vitamin E, DHA content was significantly augmented, rising to 50% of total FAs (Table 1). DHA accumulation in BMP was further enhanced after the addition of vitamin E, reaching 85% of total FAs. As reported in **Table 2**, incubation with  $30 \mu\text{M}$  22:6/22:6-PG also promoted DHA enrichment in PE, although to a lower extent, inasmuch as the proportion of



**Fig. 2.** Variation of BMP, PC, and PE amounts after supplementation with 22:6/22:6-PG. RAW macrophages were cultured in the absence or presence of  $10 \mu\text{M}$  or  $30 \mu\text{M}$  22:6/22:6-PG with or without vitamin E for 24 h. PLs were quantified as described in the legend to Fig. 1. Data represent the means  $\pm$  SD from at least four independent determinations. A, B, C: significantly different groups (multiple means comparisons by ANOVA and Tukey-Kramer method with  $\alpha = 0.05$ ). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$  versus control (one-sample *t*-test).

DHA did not exceed 30%. There was no further increase in DHA proportion in this PL in the presence of vitamin E. Similar observations were made for PC (not shown).

The protective effect of vitamin E suggests the involvement of lipid peroxidation in the mechanism of BMP decrease in response to high concentrations of 22:6/22:6-PG. After incubation with  $30 \mu\text{M}$  22:6/22:6-PG, the level of cellular MDA, an index of total lipid peroxidation, was about 1.6-fold higher than in controls (Fig. 3). The augmentation of cellular MDA was completely abolished in the presence of vitamin E. This supports the fact that prevention of BMP decrease by vitamin E was due to its antioxidant action rather than to other described effects (37). The specific peroxidation of PE was evaluated by measuring the proportion of plasmalogens (alkenylacyl subclass) in this PL. Total plasmalogen content was assessed as the sum of fatty aldehyde DMAs (16:0-DMA, 18:0-DMA, and 18:1-DMA), which are obtained after derivatization of *sn*-1 fatty alkenyls contained in plasmalogens. As indicated in Table 2, DMAs in PE were not significantly decreased during 22:6/22:6-PG supplementation, indicating no peroxidation of this PL. Also, no changes in FA composition could be observed in the presence of vitamin E, which confirms the absence of peroxidation of this highly unsaturated PL class.

#### Supplementation with nonesterified DHA induced a decrease of BMP content related to its high DHA enrichment

The incorporation of DHA in BMP when using 22:6/22:6-PG may be favored, compared with other PLs, inasmuch as PG liposomes are likely to follow the endocytic pathway and reach the late endosomes where BMP is

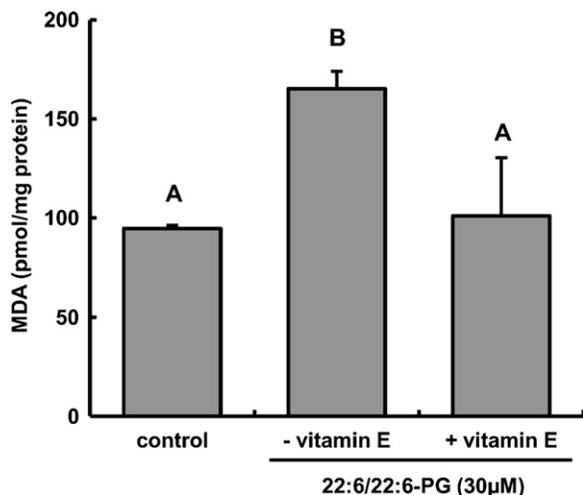
TABLE 2. FA composition of PE

FA	Control	18:1/18:1-PG (30 $\mu$ M)	22:6/22:6-PG (30 $\mu$ M)		DHA (30 $\mu$ M)	
			-Vitamin E	+Vitamin E	-Vitamin E	+Vitamin E
			<i>mol %</i>			
14:0	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1
16:0	7.3 $\pm$ 1.0	6.1 $\pm$ 0.6	6.5 $\pm$ 0.8	6.1 $\pm$ 0.9	6.9 $\pm$ 0.4	6.9 $\pm$ 1.1
18:0	14.6 $\pm$ 1.0	15.3 $\pm$ 2.0	17.9 $\pm$ 1.4	17.6 $\pm$ 2.3	17.4 $\pm$ 1.5	17.9 $\pm$ 2.8
16:1n-9/n-7	3.9 $\pm$ 0.7	2.4 $\pm$ 0.3	2.3 $\pm$ 0.5	1.8 $\pm$ 0.1	1.8 $\pm$ 0.3	1.4 $\pm$ 0.4
18:1n-9	<b>25.4</b> $\pm$ 1.9	<b>31.9</b> $\pm$ 4.8 <sup>a</sup>	13.3 $\pm$ 1.5	13.3 $\pm$ 1.6	14.3 $\pm$ 2.2	13.9 $\pm$ 2.7
18:1n-7	6.3 $\pm$ 0.9	4.2 $\pm$ 0.1	4.0 $\pm$ 0.5	3.8 $\pm$ 0.3	3.6 $\pm$ 0.6	3.3 $\pm$ 0.6
18:2n-6	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1	0.9 $\pm$ 0.2	0.8 $\pm$ 0.0	0.4 $\pm$ 0.1	0.8 $\pm$ 0.1
20:2n-6	4.3 $\pm$ 0.7	7.3 $\pm$ 2.4	tr	tr	0.8 $\pm$ 0.1	0.9 $\pm$ 0.2
20:4n-6	9.5 $\pm$ 1.4	8.7 $\pm$ 0.3	6.0 $\pm$ 0.7	6.1 $\pm$ 0.7	5.7 $\pm$ 1.4	6.3 $\pm$ 1.4
20:5n-3	1.1 $\pm$ 0.2	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0.5 $\pm$ 0.2
22:5n-3	3.7 $\pm$ 0.6	2.8 $\pm$ 0.1	2.0 $\pm$ 0.2	2.1 $\pm$ 0.3	1.9 $\pm$ 0.3	1.8 $\pm$ 0.5
22:6n-3	<b>3.9</b> $\pm$ 0.6	2.8 $\pm$ 0.1	<b>26.3</b> $\pm$ 1.4 <sup>a</sup>	<b>25.7</b> $\pm$ 0.9 <sup>a,ns</sup>	<b>30.1</b> $\pm$ 2.6 <sup>a</sup>	<b>28.9</b> $\pm$ 5.2 <sup>a,ns</sup>
16:0-DMA	10.5 $\pm$ 1.5	9.1 $\pm$ 1.0	9.8 $\pm$ 1.4	11.2 $\pm$ 1.3	9.3 $\pm$ 1.5	8.6 $\pm$ 0.6
18:0-DMA	5.6 $\pm$ 0.6	4.6 $\pm$ 0.7	5.7 $\pm$ 0.7	5.9 $\pm$ 0.5	4.7 $\pm$ 0.1	4.8 $\pm$ 0.3
18:1-DMA	3.1 $\pm$ 0.5	3.9 $\pm$ 0.8	3.4 $\pm$ 0.8	2.6 $\pm$ 0.3	2.6 $\pm$ 0.4	2.2 $\pm$ 0.3

PE, phosphatidylethanolamine; DMA, dimethyl acetal; ns, not significant; tr, trace. RAW macrophages were cultured for 24 h in the absence or presence of 30  $\mu$ M 18:1/18:1-PG, 30  $\mu$ M 22:6/22:6-PG, or 30  $\mu$ M DHA, with or without vitamin E for the two later conditions. After total lipid extraction and separation by TLC/HPLC, PE was analyzed for FA composition by GC as described in Experimental Procedures. Data are expressed as mole percent and represent means  $\pm$  SD from at least three independent determinations. Minor FAs (less than 0.5% of total) were included for calculation but not tabulated. Significant differences are only reported for bold values. ns: not significant versus the respective condition without vitamin E (two-sample *t*-test,  $\alpha = 0.05$ ). No significant difference was observed for DMA.

<sup>a</sup> *P*  $\leq$  0.05 versus control (two-sample *t*-test).

concentrated. It has been suggested that deacylation of PG, which is the first step in BMP synthesis, occurs in the endosomal compartment (38). This particular delivery pathway for 22:6/22:6-PG could determine the specific decrease of BMP if the mechanism involved was restricted to the endosomal compartment. We thus examined whether



**Fig. 3.** Cellular malondialdehyde (MDA) after supplementation with 30  $\mu$ M 22:6/22:6-PG. RAW macrophages were incubated in the absence or presence of 30  $\mu$ M 22:6/22:6-PG with or without vitamin E for 24 h. Cellular MDA levels were determined by HPLC as detailed in Experimental Procedures. Data represent means  $\pm$  SD from four wells in one representative experiment, repeated three times, with similar differences between the three conditions. A, B: significantly different groups (multiple means comparisons by ANOVA and Tukey-Kramer method with  $\alpha = 0.05$ ).

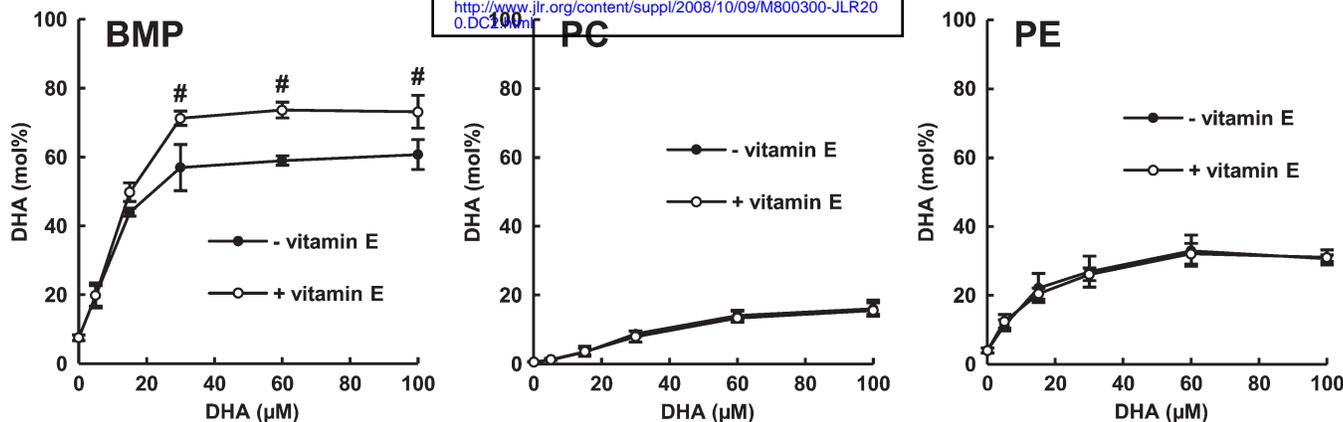
supplementation with nonesterified DHA would induce similar changes in BMP.

As expected, incubation of RAW macrophages with increasing doses of DHA induced a dose-dependent enrichment of DHA in all PLs (**Fig. 4**). However, DHA was preferentially incorporated into BMP compared with PC and PE, in agreement with our previous report in THP-1 macrophages (19). The proportion of DHA increased up to 60% in BMP compared with less than 35% in PE and 20% in PC. Moreover, the efficiency of incorporation was much higher in BMP than in other PLs, as evidenced from the slope of the dose-response curves. Of note, addition of vitamin E to  $\geq 30$   $\mu$ M DHA led to a further significant increase of DHA content in BMP (**Fig. 4** and **Table 1**), whereas the proportion remained unchanged in both PE and PC (**Fig. 4** and **Table 2**).

As shown in **Fig. 5**, the amount of BMP was unchanged after incubation with 5  $\mu$ M and 15  $\mu$ M DHA but was significantly decreased by about 50%, in the presence of  $\geq 30$   $\mu$ M DHA, when DHA represented more than 50% of the total FAs in BMP. At these concentrations, DHA significantly enhanced lipid peroxidation, as evidenced by high MDA levels compared with controls (not shown). Again, the addition of vitamin E prevented the loss of BMP.

By contrast, supplementation with nonesterified DHA did not alter PC and PE content even at the highest dose, and addition of vitamin E had no effect (not shown). As indicated in **Table 2**, both DMA and polyunsaturated FA content in PE were not changed after supplementation with 30  $\mu$ M nonesterified DHA, and the addition of vitamin E had no effect, indicating no peroxidation of this PL.

These results therefore confirm that BMP may be specifically affected during high DHA supplementation, possi-



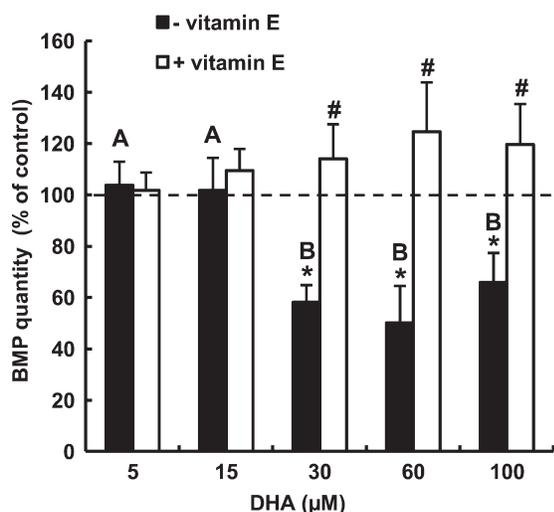
**Fig. 4.** Variation of docosahexaenoic acid (DHA) proportion in BMP, PC, and PE after supplementation with nonesterified DHA. RAW macrophages were cultured in the presence of increasing concentrations of DHA with or without vitamin E for 24 h. After total lipid extraction and separation by TLC/HPLC, PLs were analyzed for FA composition by GC as described in Experimental Procedures. Data are expressed as mole percent of DHA and represent the means  $\pm$  SD from at least three independent determinations. #  $P \leq 0.05$  versus the respective condition without vitamin E (two-sample *t*-test).

bly as a consequence of high DHA incorporation in this PL and through a peroxidation-related mechanism.

#### Analysis of BMP molecular species

When RAW macrophages were incubated with 30  $\mu$ M 22:6/22:6-PG in the presence of vitamin E, BMP accumulated, and DHA proportion averaged 85%, consistent with the formation of diDHA molecular species (Table 1). We then looked for a possible relationship between the formation of 22:6/22:6-BMP and the loss of the amount of

BMP. In control cells, the major molecular species of BMP was 18:1/18:1-BMP, as expected from the FA composition (Table 3). DHA was mainly recovered in 18:1/22:6-BMP molecular species, which represented 10% of total molecular species. Also detected in a tiny amount was 22:6/22:6-BMP. After incubation with 22:6/22:6-PG in the presence of vitamin E, the proportion of 22:6/22:6-BMP was markedly increased, by 30-fold, compared with 11-fold for 22:5/22:6-BMP and 2-fold for 18:1/22:6-BMP. These molecular species were also increased above control values when cells were incubated with 22:6/22:6-PG in the absence of vitamin E. However, although 18:1/22:6-BMP



**Fig. 5.** Variation in the amount of BMP after supplementation with nonesterified DHA. RAW macrophages were cultured in the absence or presence of increasing concentrations of DHA with or without vitamin E for 24 h. BMP was quantified as described in the legend to Fig. 1. Data represent the means  $\pm$  SD from at least three independent determinations. A, B: significantly different groups (multiple means comparisons by ANOVA and Tukey-Kramer method with  $\alpha = 0.05$ ). \*  $P \leq 0.05$  versus control (one-sample *t*-test). #  $P \leq 0.05$  versus the respective condition without vitamin E (two-sample *t*-test).

TABLE 3. Molecular species of BMP

Molecular Species	Control	22:6/22:6-PG (30 $\mu$ M)	
		-Vitamin E	+Vitamin E
		<i>mol %</i>	
16:1/18:1	5.2	1.8	0.0
16:0/18:1	4.4	1.5	0.0
18:1/18:2	6.0	1.8	0.6
18:1/18:1	32.8	10.3	4.0
18:0/18:1	8.0	3.1	1.0
16:1/22:6	1.2	2.0	1.6
18:0/22:6 and 18:1/22:5	1.7	2.6	2.0
18:1/20:4 and 16:0/22:5	2.7	1.7	0.7
18:1/20:3	4.0	1.5	0.0
18:1/20:2	6.1	1.9	0.7
18:1/20:1	4.5	1.8	0.0
18:1/22:6	10.0	22.5	21.3
18:1/22:5	6.6	6.6	6.5
18:1/22:4	3.0	1.7	0.9
22:6/22:6	1.5	29.2	44.8
22:5/22:6	1.3	8.0	14.0
22:5/22:5 and 22:4/22:6	1.2	2.0	2.1

RAW macrophages were cultured in the absence or presence of 30  $\mu$ M 22:6/22:6-PG with or without vitamin E for 24 h. After total lipid extraction and separation by TLC/HPLC, analysis of BMP molecular species was performed by electrospray MS as described in Experimental Procedures. Data are expressed as mole percent and are representative of two independent experiments.

reached similar proportions in both the absence and the presence of vitamin E, 22:6/22:6-BMP and 22:5/22:6-BMP were significantly lower in the absence of vitamin E. The conversion of percentage values to mass values for each molecular species (as calculated from total amount of BMP) indicates that addition of vitamin E triggered a huge accumulation of 22:6/22:6-BMP (+4.4 nmol/mg protein vs. 22:6/22:6-PG alone) compared with 22:5/22:6-BMP (+1.4 nmol/mg protein) and 18:1/22:6-BMP (+2.0 nmol/mg protein). This suggests that in the absence of vitamin E, 22:6/22:6-BMP species could be preferentially degraded. We may therefore propose that the decrease in the amount of BMP observed in the absence of vitamin E is partly due to the degradation of 22:6/22:6-BMP produced from 22:6/22:6-PG or by the efficient incorporation of nonesterified DHA.

#### Formation of 22:6/22:6-BMP in RAW macrophages enriched with vitamin E and in mouse liver

Our observations clearly indicate that 22:6/22:6-BMP accumulated in RAW macrophages only when vitamin E was added in 22:6/22:6-PG liposomes. We thus aimed to verify that the occurrence of diDHA molecular species of BMP in RAW macrophages was not restricted to these experimental conditions. RAW macrophages were cultured in medium supplemented with 5  $\mu$ M vitamin E as a more physiologically relevant and efficient way to provide this compound to the cells (39). After 1 week of supplementation, cellular vitamin E content was significantly increased compared with nonsupplemented cells ( $34 \pm 4$  pmol/mg protein vs.  $<1$  pmol/mg protein, mean  $\pm$  SD of four independent determinations). There was no change in the amount of BMP or BMP FA composition under these conditions, ruling out the possibility that vitamin E itself could stimulate BMP synthesis and/or DHA esterification into BMP (not shown).

After incubation with 30  $\mu$ M 22:6/22:6-PG, BMP content was augmented by about 4-fold ( $390 \pm 119\%$  of controls, mean  $\pm$  SD of four independent determinations), as observed when vitamin E was added in the liposomes. Moreover, the extent of DHA enrichment was similar, with DHA reaching  $77 \pm 10\%$  of total FAs in BMP.

In contrast to what was observed for 22:6/22:6-PG, supplementation with vitamin E did not improve the BMP accumulation elicited by 18:1/18:1-PG ( $370 \pm 101\%$  of controls, mean  $\pm$  SD of four independent determinations) or OA enrichment ( $72 \pm 1\%$  of total FAs in BMP), compared with nonsupplemented cells. Also, supplementation with vitamin E had no effect on PE content or PUFA composition under any conditions (controls, 18:1/18:1- or 22:6/22:6-PG, not shown). These results suggest that vitamin E specifically protects DHA-rich BMP.

It was also important to examine whether DHA-rich BMP, and possibly 22:6/22:6-BMP, could be produced in vivo. Although numerous previous studies have reported that dietary supplementation of DHA increased the amount of DHA in several PL classes or subclasses in many different organs and tissues, no data have been reported for BMP. To that purpose, mice were fed a standard diet (control) or a DHA-enriched diet for 4 to 32 days. There was no difference in body weight or liver weight between the high-DHA and the respective control group. The incorporation of DHA was determined in liver BMP, PC, and PE. As reported in **Table 4**, DHA represented more than 30% of total FAs in BMP of mice fed a standard diet. This FA was present in much lower proportions in PE and PC, 20% and 8%, respectively. After feeding a DHA-enriched diet for 32 days, the proportion of DHA in BMP was increased by more than 2-fold, reaching 70% of total FAs. The content of DHA was also augmented in PC and PE, without exceeding 30% and 15% of total FAs, respectively. Similar

TABLE 4. FA composition of mice liver BMP, PE, and PC

FA	BMP		PE		PC	
	Control	DHA	Control	DHA	Control	DHA
	<i>mol %</i>					
14:0	0.5 $\pm$ 0.1	0.2 $\pm$ 0.1	tr	tr	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1
16:0	15.2 $\pm$ 2.6	6.6 $\pm$ 1.8	17.4 $\pm$ 0.5	20.4 $\pm$ 0.6	28.8 $\pm$ 1.4	32.9 $\pm$ 0.9
18:0	8.2 $\pm$ 1.0	3.3 $\pm$ 1.0	17.1 $\pm$ 2.5	16.5 $\pm$ 1.6	11.9 $\pm$ 2.2	9.6 $\pm$ 0.6
16:1n-9/n-7	1.6 $\pm$ 0.3	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.9 $\pm$ 0.2	1.1 $\pm$ 0.1
18:1n-9	12.5 $\pm$ 1.3	7.7 $\pm$ 1.2	7.8 $\pm$ 0.9	6.6 $\pm$ 0.5	5.5 $\pm$ 0.6	7.3 $\pm$ 0.7
18:1n-7	1.4 $\pm$ 0.3	0.8 $\pm$ 0.3	1.4 $\pm$ 0.3	0.9 $\pm$ 0.3	1.9 $\pm$ 0.4	1.1 $\pm$ 0.2
18:2n-6	14.9 $\pm$ 1.7	5.1 $\pm$ 1.4	6.5 $\pm$ 0.9	5.3 $\pm$ 0.7	19.1 $\pm$ 1.9	20.7 $\pm$ 1.7
20:3n-6	1.3 $\pm$ 0.2	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	1.8 $\pm$ 0.3	3.0 $\pm$ 0.2
20:4n-6	10.3 $\pm$ 2.0	2.0 $\pm$ 0.5	25.7 $\pm$ 0.6	12.6 $\pm$ 0.3	21.3 $\pm$ 2.0	8.5 $\pm$ 2.5
20:5n-3	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1	0.1 $\pm$ 0.1	1.8 $\pm$ 0.5	0.1 $\pm$ 0.1	0.9 $\pm$ 0.2
22:5n-3	0.8 $\pm$ 0.3	1.2 $\pm$ 0.3	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
22:6n-3	<b>31.9 <math>\pm</math> 3.4</b>	<b>70.7 <math>\pm</math> 4.2<sup>a</sup></b>	<b>19.5 <math>\pm</math> 0.5</b>	<b>32.3 <math>\pm</math> 0.5<sup>a</sup></b>	<b>7.5 <math>\pm</math> 0.4</b>	<b>14.3 <math>\pm</math> 0.6<sup>a</sup></b>
16:0-DMA	nd	nd	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	nd	nd
18:0-DMA	nd	nd	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	nd	nd
18:1-DMA	nd	nd	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	nd	nd

nd, not detectable. Mice were fed a control or DHA-enriched diet for 32 days. After total lipids extraction from liver homogenate, BMP, PC, and PE were analyzed for FA composition as described in Experimental Procedures. Data are expressed as mole percent and represent means  $\pm$  SD from four independent determinations. Minor FAs (less than 0.5% of total) were included for calculation but not tabulated. Significant differences are only reported for bold values.

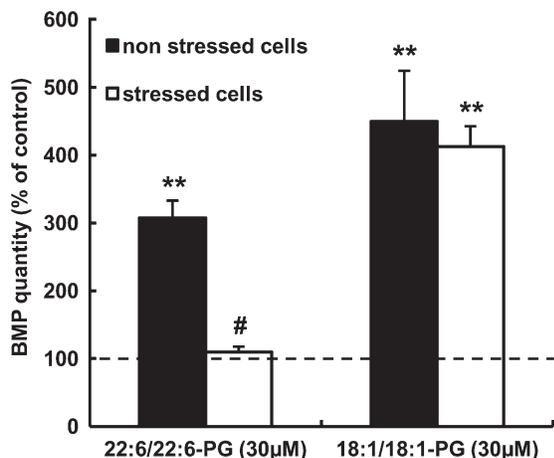
<sup>a</sup>  $P \leq 0.05$  versus the respective control (two-sample *t*-test).

changes were obtained after 4 and 8 h of diet (not shown). Of note, the data indicate that only BMP accumulated a very high proportion of DHA, consistent with the formation of diDHA molecular species.

### Preferential degradation of DHA-rich BMP and reduction of cholesterol oxidation

Altogether, our results support the idea that the decrease of BMP observed during supplementation with high concentrations of 22:6/22:6-PG (30  $\mu$ M) or nonesterified DHA ( $\geq 30$   $\mu$ M) is related to its high capacity to accumulate DHA, promoting the formation of DHA-rich BMP, especially diDHA molecular species, that could be a privileged target for peroxidation. The sensitivity of cellular DHA-rich BMP versus OA-rich BMP to oxidative stress was therefore examined. To that purpose, macrophages routinely cultured in vitamin E-supplemented medium were preincubated with 30  $\mu$ M 22:6/22:6-PG or 18:1/18:1-PG before exposure to oxidant conditions. As reported above, after incubation with 30  $\mu$ M 22:6/22:6-PG, the amount of BMP was 3-fold higher than in controls (Fig. 6), and the DHA proportion in BMP reached about 80% of total FAs (not shown). When these cells were then exposed to oxidant conditions, the amount of BMP was significantly decreased down to basal values and the proportion of DHA in BMP declined to 50% (not shown). By contrast, OA-rich BMP that accumulated after 18:1/18:1-PG incubation was not affected by oxidant conditions (Fig. 6).

One attractive hypothesis is that under oxidant conditions, degradation of DHA-rich BMP could delay or lower peroxidation of neighboring lipids, therefore acting as a local endogenous antioxidant, similar to what has been



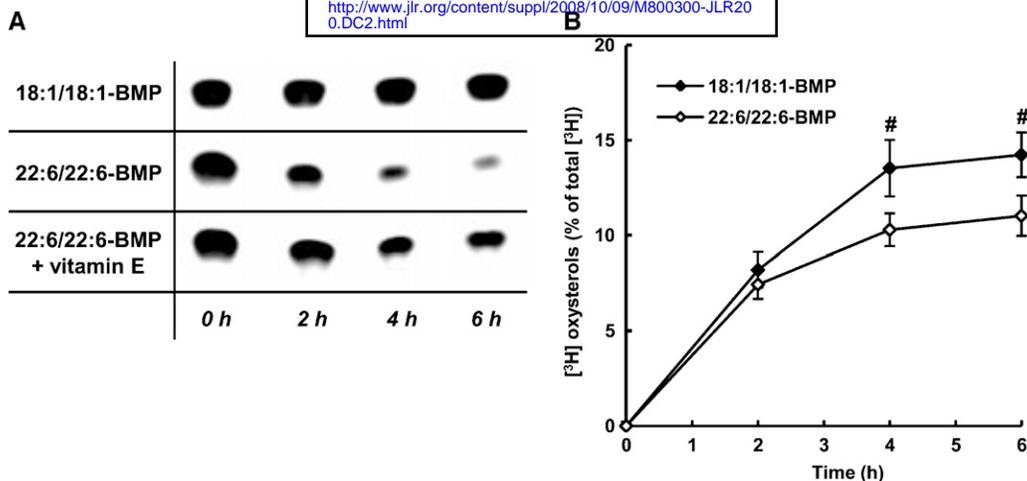
**Fig. 6.** Variation in the amount of BMP relative to oleic acid and DHA content under cell exposure to oxidant conditions. RAW macrophages routinely cultured in vitamin E-supplemented medium were preincubated with 30  $\mu$ M 18:1/18:1-PG or 22:6/22:6-PG for 24 h before exposure to oxidant conditions, as detailed in Experimental Procedures. BMP was quantified as described in the legend to Fig. 1. Data represent the means  $\pm$  SD from at least three independent determinations. \*\*  $P \leq 0.01$  versus control (one-sample  $t$ -test). #  $P \leq 0.05$  versus the respective condition in nonstressed cells (two-sample  $t$ -test).

proposed for PE plasmalogen (40). Among these lipids, cholesterol is of special interest, inasmuch as it is closely associated with BMP during LDL endocytosis and intracellular cholesterol trafficking. To test this hypothesis, a series of experiments were conducted in a cell-free system. We have compared the oxidation of cholesterol in the presence of 18:1/18:1-BMP or 22:6/22:6-BMP in reconstituted liposomes (16:0/16:0-PC/BMP/cholesterol) containing a tracer dose of radioactive cholesterol to follow the proportion of [ $^3$ H]cholesterol converted into [ $^3$ H]oxysterols. As shown in Fig. 7A, exposure to oxidant conditions induced a time-dependant degradation of 22:6/22:6-BMP that was almost complete after 6 h treatment and was much delayed in the presence of vitamin E. By contrast, 18:1/18:1-BMP remained intact, confirming the much higher oxidizability of 22:6/22:6-BMP compared with 18:1/18:1-BMP. These oxidant conditions also induced a time-dependent oxidation of cholesterol, as evidenced by the increasing proportion of [ $^3$ H]oxysterols (Fig. 7B). Of interest, the extent of oxidation was about 25% lower in liposomes containing 22:6/22:6-BMP compared with 18:1/18:1-BMP, supporting the idea that the high oxidizability of 22:6/22:6-BMP could indeed protect cholesterol from oxidation.

## DISCUSSION

It was clearly established in previous metabolic studies that exogenous PG is converted to BMP (26–28). We here further demonstrate that supplementation with 18:1/18:1-PG or 22:6/22:6-PG in the presence of vitamin E induces BMP accumulation up to 4-fold the control level. Moreover, we show that the FA composition of BMP may vary depending on the nature of the PG precursor. Waite et al. (28, 41) have postulated that the first step in BMP synthesis involves a PLA<sub>2</sub> that cleaves the 2-acyl group from PG. They later purified and characterized a novel lysosomal PLA<sub>2</sub> from RAW macrophages, and comparison using PG with various acyl chains at the *sn*-2 position revealed a high affinity toward oleate (38). This could explain the efficient synthesis of BMP from 18:1/18:1-PG, which led to a 2-fold increase in the amount of BMP at only 5  $\mu$ M. It is assumed that the next step in BMP synthesis from exogenous PG requires the reacylation of intermediate products, i.e., lyso PG, through a transacylation reaction, with PLs as acyl donors (27, 42, 43). Waite's group initially proposed a pathway for BMP synthesis in which the PG glycerol backbone was conserved but not the FAs. However, they later described a transacylase that uses two lyso PGs, one as an acyl donor and another as an acyl acceptor (44) in RAW macrophages. This activity is consistent with our observation that PG supplied not only the glycerol backbone but also the FAs for BMP formation.

PG is also known as a precursor of CL (45, 46). However, several studies suggest a complex regulation of CL synthesis, which is not dependent only on the PG substrate. It was indeed shown that a 10- to 20-fold increase in PG content did not lead to CL accumulation (47). More recently,



**Fig. 7.** Oxidation of BMP and cholesterol in a cell-free system. Liposomes reconstituted with 16:0/16:0-PC, 18:1/18:1-BMP, or 22:6/22:6-BMP and cholesterol (75:20:5) were exposed to 100  $\mu$ M  $\text{CuSO}_4$  and 10 mM  $\text{H}_2\text{O}_2$  for 2 h to 6 h at 40°C. Lipids were extracted and analyzed as detailed in Experimental Procedures. A: TLC patterns of BMP are representative of three independent experiments. B: Cholesterol oxidation was determined as the proportion of total radioactivity (free and oxidized cholesterol) associated to oxysterols. Data represent the means  $\pm$  SD from quadruplicate of one experiment, repeated three times with similar differences between 18:1/18:1-BMP- and 22:6/22:6-BMP-containing liposomes. Oxysterols were not detected before oxidation ( $t_0$ ). #  $P \leq 0.05$  versus the respective incubation time for liposomes reconstituted with 22:6/22:6-BMP (two-sample *t*-test).

we observed that increasing the synthesis of PG by phosphatidylglycerophosphate synthase overexpression was not associated with an enhanced formation of CL in CHO cells (29). Consistently, we did not measure any accumulation of CL after supplementation with 18:1/18:1-PG or 22:6/22:6-PG, confirming the absence of a tight correlation between PG and CL.

Our results indicate that supplementation with PG may provide a useful device to better understand the role of BMP in relation to its quantity and its FA composition. It is highly likely that both the stereoconfiguration and the cellular location of BMP are critical for its physiological functions. It was demonstrated that BMP produced from exogenous PG by RAW macrophages keeps the natural *sn1-sn1'* stereoconfiguration (48). Of note, similar to control cells, BMP immunofluorescence in 18:1/18:1-PG-treated cells revealed a punctuate and perinuclear staining, supporting an endosomal location (not shown). Moreover, in BHK cells in which the amount of BMP was increased by 2-fold after supplementation with 30  $\mu$ M 18:1/18:1-PG, we have confirmed by cell fractionation and lipid analysis that the majority of BMP was recovered in late endosomes and not other fractions (not shown). This indicates that BMP synthesized from exogenous PG is specifically located in late endosomes, similar to endogenous BMP (1, 3). We may then assume that supplementation of RAW macrophages with 18:1/18:1-PG increases BMP content without altering its stereoconfiguration and cellular location, providing an alternative approach to the anti-BMP antibody or drugs such as D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (8, 12) to investigate the functions of BMP in cellular models.

In agreement with previous observations in THP-1 macrophages and other cell types (17, 19, 21), we found that DHA was preferentially incorporated into BMP compared with PC and PE in RAW macrophages. We herein additionally provide detailed analysis of BMP molecular species that shows the prevalence of 22:6/22:6-BMP formation under DHA supplementation. Moreover, we report that the high avidity of BMP for DHA also occurs during *in vivo* supplementation. Most importantly, our present data clearly indicate that BMP was selectively decreased during high supplementation of RAW macrophages with DHA, provided to the cells in nonesterified form or as 22:6/22:6-PG. Although not directly demonstrated, our results suggest that oxidation of DHA-rich BMP might be the molecular reason for the reduction in cellular BMP. This conclusion is supported by the outcome of the vitamin E experiments, the increased MDA formation, and the proclivity of DHA-rich BMP and 22:6/22:6-BMP for lipid peroxidation in cellular and cell-free systems. Alternatively, DHA and 22:6/22:6-PG themselves could undergo peroxidation in the absence of vitamin E, generating lipid peroxides and oxidant species that could potentially promote specific degradation of BMP. However, this is quite unlikely because endogenous BMP (i.e., BMP of control cells) was resistant under cell exposure to various oxidative stresses (not shown). If DHA and 22:6/22:6-PG were first degraded in the absence of vitamin E, they would be less available for BMP acylation and BMP synthesis, which could explain the differences between the presence and the absence of vitamin E. But we would then also expect that addition of vitamin E would increase the incorporation of DHA in PE and PC. Another explanation for the

decrease of BMP in the absence of vitamin E, but that DHA and 22:6/22:6-PG could promote the activation of BMP-hydrolyzing enzyme, possibly through the generation of oxidant species/lipid peroxides. As a matter of fact, lysosomal phospholipase A<sub>2</sub> activity has been reported to exert hydrolytic activity on BMP, including DHA containing BMP (49). It is yet totally unknown whether this enzyme is sensitive to lipids or lipid peroxides.

Our data show that only BMP mass was decreased after DHA incorporation, while DHA was also accumulated in PC and PE, although to a lower extent. One may argue that partial degradation of PC and PE may also occur without affecting the amount of these PLs due to their initial high content compared with BMP. However, the plasmalogen content in PE was not decreased during DHA or 22:6/22:6-PG supplementation, indicating no peroxidation of this PL. Moreover, because vitamin E did not improve DHA incorporation in PC and PE, in contrast to BMP, it is unlikely that they undergo peroxidation consecutive to DHA enrichment. An important point to consider is the specific location of BMP in the endosomal compartment (1, 3). We cannot exclude that oxidative status in late endosomes is higher than in the whole cell, resulting in the specific degradation of BMP. Late endosomal/lysosomal compartments contain high levels of iron that could favor oxidation reactions (50) and exhibit oxidizing redox potential (51). It was recently suggested that macrophage lysosomes are the intracellular sites for oxidation of LDL in macrophages (52). Another likely explanation is the difference in the formation of diDHA molecular species. Based on the DHA proportion recovered in PE and PC, diDHA molecular species of these PLs probably reached only moderate levels compared with BMP. DiDHA molecular species of PC, PE, and phosphatidylserine have been reported in the retina of different animals, including rat, bovine, and dog (53–55). Despite very high percentages of DHA in retina PL, diDHA species do not exceed 10% of total DHA-containing species, even after DHA supplementation. The reason that BMP highly accumulates DHA and is able to form such high levels of diDHA molecular species is not understood. This may be related to its very peculiar structure. Indeed, in contrast to other PLs, BMP binds its two FAs on two different glycerol moieties (15, 16). It has been reported that increasing proportions of DHA in lipids augments their vulnerability to peroxidation (56, 57). Moreover, the presence of DHA at all positions, compared with only one position, was shown to increase the oxidizability of lipids such as triacylglycerols and PC (58). In rat photoreceptor membranes, diDHA PL molecular species are selectively decreased under oxidative stress related to light (59).

Several studies have reported a correlation between vitamin E levels and cellular redox status (60, 61). In monocytes, vitamin E was shown to decrease the release of reactive oxygen species and lipid oxidation (62). It has been stipulated that most cultured cells are deficient in vitamin E, resulting in increased free radical-mediated lipid peroxidation (63). Thus, the relative deficiency in vitamin E in nonsupplemented macrophages could pro-

mote pro-oxidant status in these cells, therefore favoring the degradation of 22:6/22:6-BMP, in contrast to OA-rich BMP that was not affected by vitamin E level. In macrophages supplemented with vitamin E, DHA-rich BMP was protected from degradation. Of interest, cellular vitamin E in supplemented macrophages reached physiologically relevant levels when compared with those reported in primary monocytes/macrophages (64), suggesting that the formation of DHA-rich BMP may also occur in these cells under DHA supplementation. In mouse liver, BMP accumulated very high proportions of DHA, consistent with the formation of 22:6/22:6-BMP, but the content of the PL was not decreased (not shown). This may be related to the high level of vitamin E in this organ, especially in the endosomal/lysosomal compartment (37).

Despite great interest to support our hypothesis that the decrease of BMP is due to DHA-rich BMP peroxidation, we did not attempt to further elucidate the mechanism involved in BMP degradation or to characterize the degradation products. Under our analytical conditions (TLC, HPLC), we did not detect any additional spot or peak that could correspond to oxidized forms of BMP in cell lipid extracts. After total oxidation of 22:6/22:6-BMP in a cell-free system, as evidenced by the almost complete disappearance of the spot corresponding to BMP on the TLC plate, no other spot and no smear could be detected. These observations suggest that many different degradation products might be formed, in levels too low to allow their detection under our conditions. Studies by Salomon et al. (65, 66) showed that cleavage of DHA-containing PC and PE by free radical-induced oxidative stress generates a complex mixture of oxidatively truncated PLs, some of whom react with lysyl residue to form peptide adducts. Their production was described in a cell-free system, and their natural occurrence was reported in the retina and in atherosclerotic plaques. Whether DHA-containing BMP can produce analogous structures of oxidatively truncated BMP surely merits further examination, although it may be a rather complicated issue.

Many studies on cultured cells and dietary supplementation, including some from our laboratory, have reported that high DHA could be deleterious, mainly because it may promote cellular oxidative stress. Our results indicate that high DHA supplementation may exert toxic effects on BMP in relation to high incorporation of DHA, with potential consequences on endosomal/BMP-related functions. On the other hand, the high capacity of BMP to incorporate DHA could be beneficial to cells because it could prevent excessive accumulation of nonesterified DHA. Of note, our data show that DHA-rich BMP, especially 22:6/22:6-BMP, are very prone to oxidative degradation compared with OA-rich BMP and 18:1/18:1-BMP. The high sensitivity of 22:6/22:6-BMP could make this molecular species a potential antioxidant in its immediate vicinity. Of particular interest, the degradation of 22:6/22:6-BMP was associated with a significantly lower oxidation of cholesterol in a cell-free system. This is relevant because cholesterol is closely associated with BMP in late endosomes during LDL endocytosis. It was reported that oxidation

of LDL-derived cholesterol can occur intracellularly in macrophages, most probably within lysosomes (52). BMP was identified as one of the antigenic targets in anti-PL syndrome (1, 14, 62). Of potential interest, enhanced lipid peroxidation has been proposed to play an important role in the pathogenesis of anti-PL syndrome (14). Independently of DHA supplementation, BMP from uterine stromal cells, rat liver, and mice liver was reported to contain a high content of DHA (17, 20, and present data). Moreover, 22:6/22:6-BMP was specifically accumulated in rat liver lysosomes (67) and recovered in urine after treatment with different phospholipidosis-inducing drugs (68). Our observations open new perspectives on the functional significance of the high avidity of BMP for DHA. The potential antioxidant action of DHA-rich BMP deserves particular attention. ■■

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