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The Formation of Phosphatidylcholine Oxidation Products by Stimulated Phagocytes

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Phagocytic cells produce a variety of oxidants as part of the immune defence, which react readily both with proteins and lipids, and could contribute to the oxidation of low density lipoprotein in atherosclerosis. We have investigated the oxidation of phospholipid vesicles by neutrophils and mononuclear cells, to provide a model of lipid oxidation in the absence of competing protein. Phorbol 12-myristate 13-acetate-stimulated neutrophils were incubated with phospholipid vesicles containing dipalmitoyl phosphatidylcholine, palmitoyl-arachidonoyl phosphatidylcholine (PAPC) and stearoyl-oleoyl phosphatidylcholine, before extraction of the lipids for analysis by HPLC coupled to electrospray mass spectrometry. The formation of monohydroperoxides (814 m/z) and bis-hydroperoxides (846 m/z) of PAPC was observed. However, the major oxidized product occurred at 828 m/z, and was identified as 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine. These products were also formed in incubations where the neutrophils were replaced by mononuclear cells, and the amounts produced per million cells were similar. These results show that following oxidative attack by phagocytes stimulated by PMA, intact phospholipid oxidation products can be detected. The identification of an epoxyisoprostane phospholipid as the major product of phagocyte-induced phospholipid oxidation is novel, and in view of its inflammatory properties has implications for phagocyte involvement in atherogenesis.

Keywords: Atherosclerosis; Electrospray ionization mass spectrometry; Neutrophils; Oxidative damage; 1-Palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine; Phospholipid hydroperoxide

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; ESMS, electrospray mass spectrometry; LDL, low density lipoprotein; MNCs, mononuclear cells; m/z, mass-to-charge ratio; PAPC, palmitoyl-arachidonoyl phosphatidylcholine; PC, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes; SOPC, stearoyl-oleoyl phosphatidylcholine

INTRODUCTION

Phagocytic cells such as neutrophils, monocytes and macrophages are an important part of the innate immune response, which is in part owing to their ability to produce cytotoxic and microbicidal reactive oxygen species, including superoxide (O_2^-), hydrogen peroxide, singlet oxygen, nitric oxide and hypochlorous acid. These can cause oxidative damage to biomolecules, including proteins and lipids,[1] and could, therefore, have a role in the oxidation of LDL, which is accepted to play a key role in the development of atherosclerosis.[2,3] Oxidative modification of LDL results in its uncontrolled uptake by macrophages, monocytes and smooth muscle cells via scavenger receptors, leading to foam cell formation and the development of a fatty streak.[4,5] Thus information on the ability of phagocytic cells to induce lipid oxidation and the types of oxidation products that result would be essential to an understanding of the potential role of these cells in the initiation and progression of atherosclerosis.

There are a number of reports establishing that activated human phagocytes can cause oxidation of lipoproteins. Cathcart *et al.*[6] showed that human monocytes and polymorphonuclear leukocytes

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(PMNs) can peroxidize LDL, rendering it cytotoxic to fibroblasts, and that this involves the initial release of superoxide followed by superoxide-independent propagation of oxidation.[7] Steinbrecher[8] also concluded that superoxide was an essential, though possibly indirect, mediator of the peroxidation of LDL induced by various cell types. However, in the majority of these studies lipid peroxidation has been assessed by the measurement of thiobarbituric acid reactive substances, which detects aldehyde breakdown products of polyunsaturated lipid peroxides (e.g. malondialdehyde) and is thus a relatively indirect and uninformative method. Very few studies have utilized methods that detect a variety of lipid oxidation products, such as HPLC or GC-MS. The effect of human PMNs on dilinoleoyl phosphatidylcholine (DLPC) liposomes was monitored by reverse phase HPLC with detection at 234 nm and GC-MS, and was found to generate 9- and 13-hydroperoxy derivatives of linoleic acid.[9] Similar results were obtained for mouse macrophages incubated with LDL and ^{14}C-labelled linoleic acid, as analysis by HPLC showed the presence of products which co-eluted with 13- and 9-hydroxyoctadecadienoic acids.[10] These reports provide more detailed information about the possible lipid oxidation products resulting from attack by activated phagocytes, but they have still focused essentially on the breakdown products of peroxidation.

LDL contains polyunsaturated fatty acids esterified in phospholipids, of which a major component is phosphatidylcholine. There is growing evidence that intact oxidized phospholipids can have biological effects; for example, 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(2-glutaryl)-*sn*-glycero-3-phosphocholine, have been found to stimulate monocyte-endothelial adhesion.[11,12] These compounds can be generated by autoxidation of palmitoyl arachidonoyl phosphatidylcholine, together with a large number of other products, but they have also been detected in lesions from rabbits fed on a high cholesterol diet, and it is possible that their presence there may play a role in the further development of atherosclerotic lesions.

As yet, it is unclear how these oxidation products are formed *in vivo*. We have therefore investigated the oxidation of phosphatidylcholine vesicles by PMA-stimulated phagocytes using liquid chromatography coupled to electrospray mass spectrometry, which allows a wide range of phospholipid oxidation products to be detected, as described previously.[13,14] This is the first report of the observation of early and intact phospholipid oxidation products following oxidation by phagocytes.

EXPERIMENTAL

Methanol and Histopaque media were purchased from Sigma-Aldrich (Gillingham, Dorset, UK); hexane was purchased from Rathburn Chemicals (Walkerburn, UK) and ammonium acetate was purchased from Merck (Poole, Dorset, UK). Phosphatidylcholines were purchased from Sigma Chemical Company, Poole, UK. All solvents and chemicals used were of analytical quality.

Preparation of Phagocytes

Venous blood was obtained from healthy consenting volunteers in EDTA tubes, to minimize leukocyte activation. Polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNCs) were prepared by density gradient centrifugation of whole blood using Histopaque-1077 and Histopaque-1119 according to the manufacturers instructions. Erythrocyte contamination was removed by hypotonic lysis in 0.83% ammonium chloride, 10 mmol/l HEPES-NaOH pH 7.0 for 6–7 min at 37°C, followed by isotonic recovery with PBS (100 mmol/l Na_2HPO_4, 25 mmol/l NaH_2PO_4, 0.9% NaCl, pH 7.2). The cells were washed twice with PBS and counted using trypan blue to assess the viability (routinely $>95\%$). The MNC preparation contained both monocytes and lymphocytes, in addition to platelets, but the number of cells used in the incubation was calculated as the number of monocytes only (counted based on morphological appearance and the ability to reduce nitroblue tetrazolium (NBT)). The cells were resuspended in PBSG (PBS containing 5 mmol/l KCl, 5 mmol/l glucose, 0.5 mmol/l $MgSO_4$) to give cell densities between 2×10^6 and 5×10^7 cells/ml. Cell preparations were used within 1 h.

Analysis of Cellular Oxidant Production

Cellular oxidant production was monitored using NBT to confirm that the cells were activated. A measured quantity of 100 μl of cell suspension (2×10^6 cells/ml) was incubated at 37°C with 100 μl of 0.3% NBT in 0.34% sucrose solution and 4 μl of PMA (50 μg/ml in DMSO). Only DMSO was used for the control-stimulated cells. Aliquots were removed at intervals and the number of stained cells was counted.

Preparation of Phospholipid Vesicles

Phospholipid vesicles containing equal amounts of di-palmitoyl phosphatidylcholine (DPPC), palmitoyl-arachidonoyl phosphatidylcholine (PAPC) and stearoyl-oleoyl phosphatidylcholine (SOPC) were prepared as described previously[15] to give a total lipid concentration of 10 mg/ml. DPPC was included

in the vesicles as a saturated lipid standard that should be resistant to oxidation, and would allow the amounts of lipids to be estimated during the analysis by mass spectrometry. PAPC was the polyunsaturated lipid on which free radical attack and hydrogen abstraction was expected, and SOPC was included as a mono-unsaturated control that should be resistant to free radical attack.

Incubation of Cells with Phospholipid Vesicles

MNC and PMN preparations were stimulated with PMA and incubated with the phospholipid vesicles for 4 h at 37°C, with gentle mixing. The complete incubation mixture (0.5 ml) contained the following concentrations of added constituents: 0.1 mg/ml phospholipids, 1 μmol/l PMA in DMSO, 2 mmol/l $CaCl_2$, 1×10^6 to 2×10^7 cells, as indicated in the figure legends. For both MNCs and PMNs, the following incubations were carried out: a complete incubation, a control without PMA, and a control without vesicles. An additional control containing all components except the cells was also performed. The incubations were terminated by the addition of an equal volume (0.5 ml) of warm methanol, and the extraction was completed as described in Ref. [14]. Each experiment was carried out five times with comparable results, and representative sets of data are shown.

Liquid Chromatography-Electrospray Mass Spectrometry

LC-MS was performed in the positive-ion mode either on a VG Platform mass spectrometer (Micromass, Altrincham, Cheshire, UK) or an LCQ Duo (Thermo-Finnigan, Hemel Hempstead, UK), with a Phenomenex Luna C_8 reverse phase column as described previously.[14] Lipid extracts were reconstituted in 100 μl 1:5 chloroform/methanol and diluted 10-fold in running solvent; 10 μl were injected per run. Peak top spectra were collected between 400 and 1000 m/z with sweep time of 2 s. Reconstructed mass chromatograms showing individual molecular species were generated using MassLynx or Xcalibur software, and are mean smoothed. Small differences in retention times between runs occurred due to variations in ambient temperature. For calculation of the amount of oxidized products, the peak regions corresponding to isomers of the same oxidized products were integrated and compared to that of DPPC in the vesicles-only control incubation, which corresponded to a known amount of lipid. The results are expressed as pmols of oxidized lipid produced per 10^6 cells in the incubation. Differences in detection sensitivity of different lipid species were not taken into account when calculating the amounts, as the differences between similar chain lengths[15] or oxidized and unoxidized chains are thought to be fairly small.

Autoxidation of PAPC and Analysis of Oxidized Products

A 50 μl aliquot of PAPC (10 mg/ml in chloroform) was dried under nitrogen and allowed to autoxidize in the dark at 40°C for 100 h. The lipid was reconstituted in 50 μl of chloroform and diluted 200-fold in LC-MS running solvent. Spectra were collected on an LCQ Duo between 400 and 1100 m/z to obtain a profile of the autoxidation products, and the retention times for the peaks at 810 and 828 m/z were determined by LC-MS using the HPLC conditions described above. The autoxidation mixture was reacted with 35 mmol/l sodium borohydride in acetonitrile for 30 min, extracted in 1:1 water/ethyl acetate, and reanalysed by ESMS to determine the number of reducible groups.

RESULTS

The production of reactive oxygen species by the MNC and PMN preparations was monitored using the formazan dye NBT to confirm that the PMA at 1 μmol/l was inducing activation and that superoxide was being formed. Both the PMN and MNC preparations gave comparable stimulation after 30 min, with 77 ± 8 and 81 ± 2% of the cells stained, respectively. Very little stimulation was observed in the control cells (without PMA): 13 ± 6% stained cells for MNCs and 9 ± 2% for PMNs, showing that the level of non-specific activation was low.

An experiment was also carried out with PMN preparations to determine whether phosphatidylcholine vesicles in the absence of PMA could cause stimulation. It was found that the vesicles were able to stimulate the release of reactive oxygen species in the absence of PMA; after 30 min the number of stained cells in incubations with the vesicles was significantly higher (22 ± 3%) than in incubations without PMA and vesicles (9 ± 3%), although lower than that obtained with PMA.

Reverse phase LC-MS provides a sensitive method for monitoring lipid oxidation, as oxidized phospholipids elute earlier than native lipids, facilitating their identification and preventing loss of signal due to ion suppression by more abundant species. The formation of oxidized phospholipids can be monitored by the appearance of signals at specific increased masses; for example, lipid hydroperoxides can be monitored by the increase in mass of 32 or multiples thereof.[16] Chain shortened phospholipids and lysolipids can also be detected by their characteristic masses and elution times

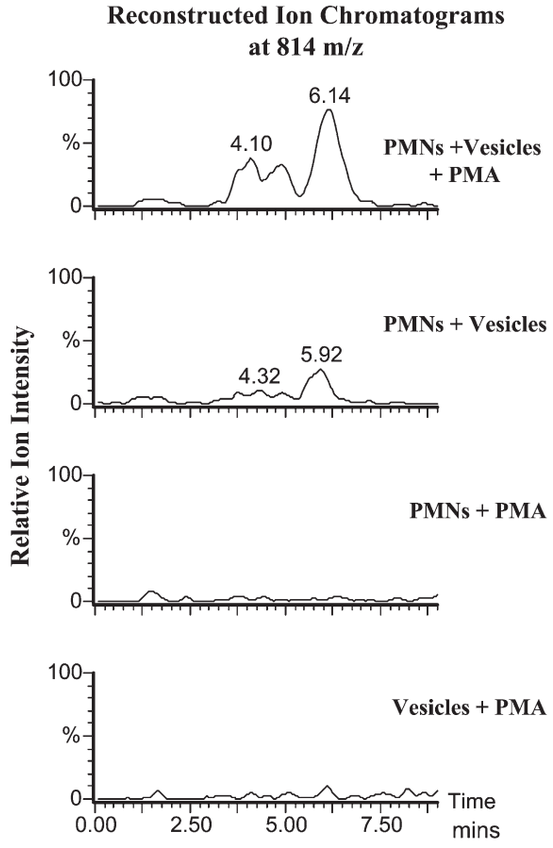

FIGURE 1 Production of a mono-hydroperoxide of PAPC by PMNs. RICs at 814 m/z, corresponding to the mass of a mono-hydroperoxide of PAPC (782 + 32), for each of the four different incubations. The vertical axes are linked to allow comparison of the peak intensity, with 100% corresponding to an ion current of 1×10^4. The complete incubation contained 1.2×10^7 PMNs/ml, 0.1 mg/ml vesicles and 1 μmol/l PMA, with PBSG as the incubation buffer. Individual components were omitted as indicated on the chromatograms; DMSO was substituted for PMA in "PMNs + vesicles". Chloroform–methanol extracts of the PMN preparations, and phospholipid vesicles without cells, were dried and reconstituted in LC solvent (71:5:7 methanol/hexane/0.1 mol/l ammonium acetate). LC was carried out using a Phenomenex Luna column with a flow rate of 0.1 ml/min. Peak top data were collected between 600 and 1000 m/z.

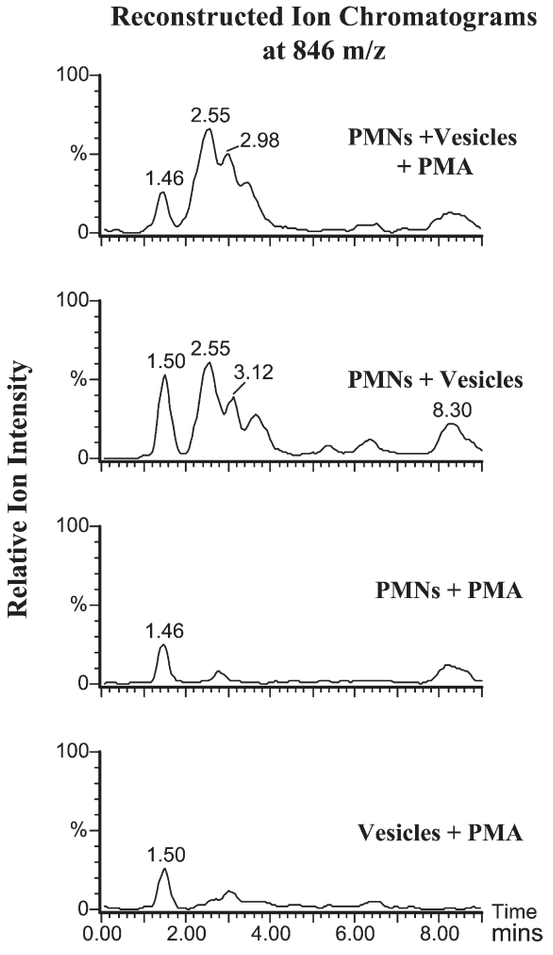

FIGURE 2 Production of a bis-hydroperoxide of PAPC by PMNs. RICs at 846 m/z, corresponding to the mass of a bis-hydroperoxide of PAPC (782 + 64), for each of the four different incubations. All experimental details are as described in Fig. 1, except for the cell concentration, which was 9×10^6 PMNs/ml. The vertical axes are linked to allow comparison of the peak intensity, with 100% corresponding to an ion current of 2×10^4.

(these compounds also elute earlier than the native lipids from which they are derived). The lipid extracts of the cell-vesicle incubations were therefore analysed by LC-MS, and the chromatograms were examined for compounds eluting early in the chromatograms at masses corresponding to hydroperoxides, hydroxides or other known products, and absent in the control treatments. For example, a mono-hydroperoxide of PAPC (native mass 782) corresponds to 814 m/z, and is apparent in the reconstructed ion chromatograms (RICs) of PMNs incubated with vesicles in the presence of PMA (Fig. 1), eluting at around 4.1 min. In fact, there are three discernible peaks eluting between 4 and 6 min, reflecting the occurrence of different isomers of the monohydroperoxide, dependent on which of the bis-allylic hydrogens was abstracted. Vesicles incubated with PMNs in the absence of PMA stimulation also produce significant monoperoxide peaks, although with slightly lower intensity, which is in agreement with the observation in experiments with NBT that vesicles themselves are able to stimulate the cells. In contrast, the controls without PMNs or without vesicles had no detectable mono-hydroperoxide. Thus it can be clearly seen that the mono-hydroperoxide is only produced in incubations containing both vesicles and cells, and must therefore be a result of phagocyte oxidation of the vesicle lipids. Similarly, RICs at 846 m/z showed the appearance of a bis-hydroperoxide of PAPC (782 + 64 m/z) in vesicles incubated in the presence of neutrophils and PMA (Fig. 2). The major peak eluted at 2.55 min, significantly earlier than the mono-hydroperoxide, as expected for a more

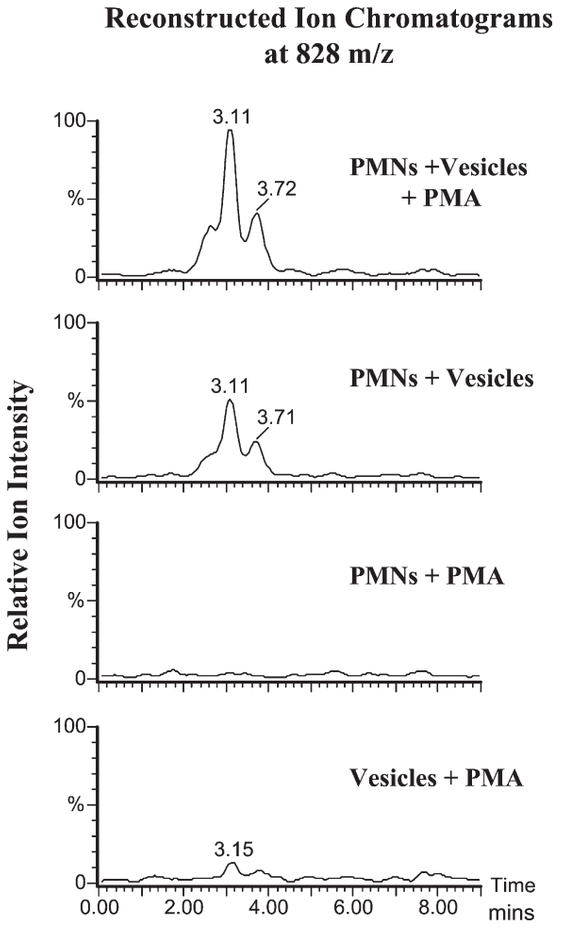

FIGURE 3 Production of an epoxyisoprostane of PAPC by PMNs. RICs at 828 m/z, corresponding to the mass of 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine, for each of the four different incubations. All experimental details are as described in Fig. 1, except the cell concentration, which was 5×10^6 PMNs/ml. The vertical axes are linked to allow comparison of the peak intensity, with 100% corresponding to an ion current of 2×10^4.

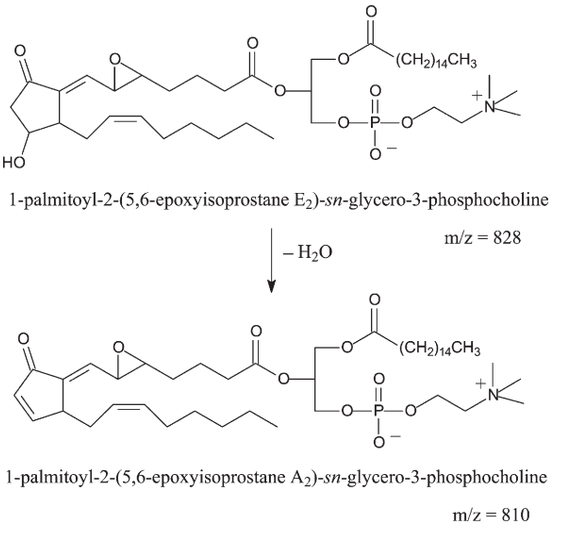

FIGURE 4 Structure of 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine and its dehydration product.[12]

oxidized product and described previously.[14] As observed for the mono-hydroperoxide, vesicles incubated with cells in the absence of PMA also showed oxidation, which, in this case, was almost comparable to that of the PMA-stimulated cells.

The chromatograms were also examined for evidence of the formation of other oxidized lipid products, such as hydroxides or peroxides of other lipids, in the incubations containing both cells and lipid vesicles. By far, the strongest signal with characteristics of an oxidized phospholipid appeared at 828 m/z. This species was present at high intensity in extracts of vesicles plus PMNs stimulated with PMA, and at a lower intensity in extracts of incubations in the absence of PMA (Fig. 3). It was completely absent in extracts of PMNs only, but very low levels were detected in extracts of vesicles without cells. This species was tentatively identified as 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine (Fig. 4), which has previously been reported as an autoxidation product of purified PAPC *in vitro*.[12] The small amount of this compound present in the vesicle extract is probably the result of autoxidation over the course of the incubation; but it can be seen that the oxidation process is enormously enhanced by the presence of the PMNs. The samples that contained the species at 828 m/z also contained a peak at 810 m/z eluting fractionally later (data not shown); this could correspond to the dehydration product of the epoxyisoprostane that has been described previously. The spectra corresponding to the early part of the chromatogram (1.5–6 min) show the relative intensities of the different oxidized products observed, and it is clear that the product at 828 m/z is the most favourable or stable product (Fig. 5).

To support the identification of this species as the epoxyisoprostane identified by Watson *et al.*[12] PAPC was autoxidized *in vitro* for comparison. The ESMS product profile obtained matched that reported previously[11] almost exactly, with major products appearing at 594, 610, 810 and 828 m/z. Fragmentation studies carried out using ion trap MSMS showed that the species at 828 m/z readily underwent dehydration to give a product at 810 m/z, which was not susceptible to dehydration. LC-MS of the autoxidized mixture showed the species at 828 m/z eluting with a very similar profile to that of the PMN-vesicle extracts; alteration of the running solvent to give longer elution times allowed better resolution of the 828 m/z peak, showing the presence of five isomers as reported previously. Treatment of the autoxidized mixture with sodium borohydride resulted in the peak at 828 m/z disappearing, and a new peak at 832 appearing, confirming the presence

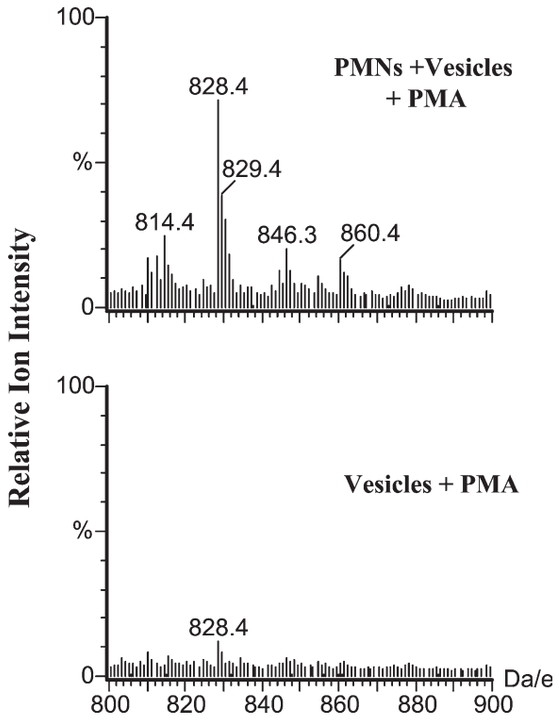

FIGURE 5 Comparison of the formation of various oxidized phospholipid products in PMNs. Spectra corresponding to 1.5–6 min of the chromatograms for stimulated neutrophils plus vesicles and PMA, or vesicles plus PMA, are evaluated showing the relative intensities of the oxidized species detected. The epoxyisoprostane phospatidylcholine is at 828 m/z and its ^{13}C-isotope peak can clearly be seen at 829 m/z; the mono-hydroperoxide of PAPC (814 m/z) and the bis-hydroperoxide of PAPC (846 m/z) are also indicated in the spectra. The product at 860 m/z was not identified.

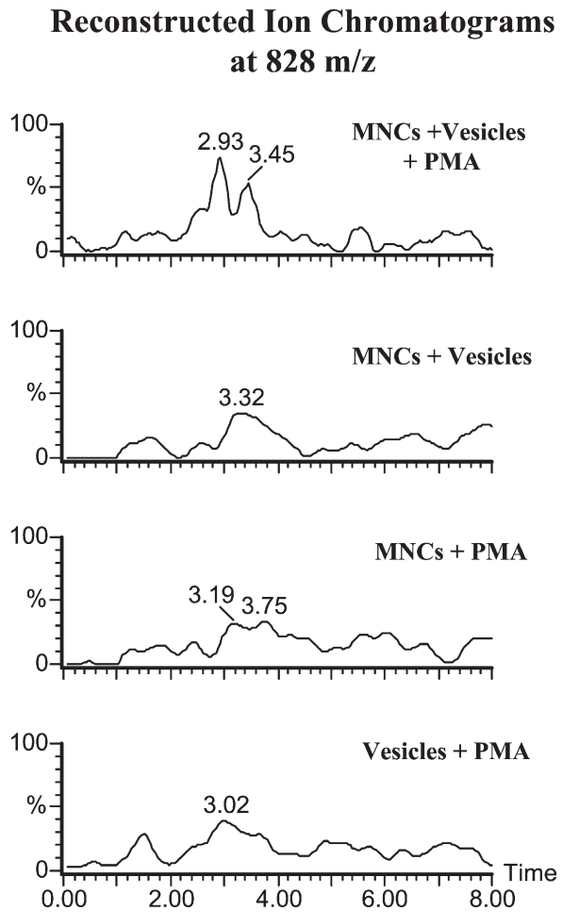

FIGURE 6 Epoxyisoprostane phosphatidylcholine formation by MNC preparations. RICs at 828 m/z from the four different incubations of MNCs (2×10^6 monocytes/ml). All other experimental details are as described in Fig. 1. The vertical axes are linked, with 100% corresponding to an ion current intensity of 3×10^3 (note the intensity is much lower than the PMN chromatograms, and that the elution times are fractionally longer than those in Fig. 3).

of two reducible groups.[12] These results support the conclusion that the species with a mass of 828 Da, generated in the presence of both PMNs and lipid vesicles, is 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine.

In parallel with the experiments on polymorphonuclear cells, incubations were also carried out with mononuclear cell (MNC) preparations to determine whether monocytes were able to oxidize the phospholipids in the same way as the neutrophils. The recovery of monocytes was much lower than that of PMNs, owing to the much lower percentage of monocytes present in peripheral blood (5–8% versus 50–70%), so the concentration of these cells was substantially lower in all the incubations. This resulted in much lower signal intensity in the chromatograms, as can be seen in Fig. 6, which shows the production of the epoxyisoprostane phospholipid at 828 m/z by MNC preparations. This product can be detected clearly in the MNC incubation with vesicles and PMA, with signals eluting at approximately 3 min, but in the absence of PMA the level was not significantly above baseline, and the levels in the controls without cells or without vesicles were also undetectable. Overall, the profile of oxidation by the MNCs was similar to that observed with the PMNs, with the epoxyisoprostane phospholipid being the major product formed, and lower levels of the hydroperoxides. In order to facilitate comparison between the two phagocytic cell populations, the amounts of epoxyisoprostane phospholipid, PAPC mono-hydroperoxide and PAPC bis-hydroperoxide were estimated by integration of the signals in the RICs, and normalized per million cells in the incubations (Fig. 7). It can be seen that the ability of the MNCs to oxidize the lipids was very comparable to that of the neutrophils; the amounts of hydroperoxides are almost identical between the two cell populations, and although the neutrophils appear to yield slightly higher amounts of the epoxyisoprostane phospholipid, the difference was not found to be significant ($p = 0.57$ according to the Student t-test).

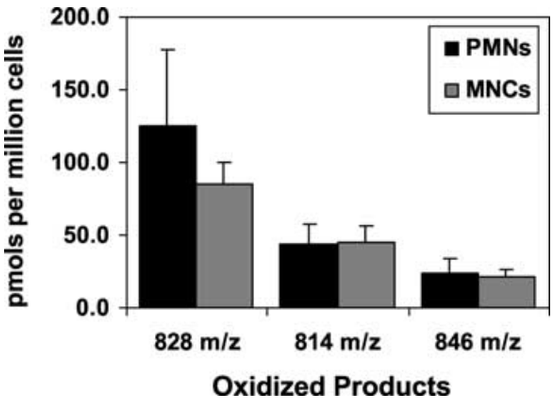

FIGURE 7 Comparison of oxidized phospholipid formation by PMN and MNC preparations. The signals corresponding to the oxidized products in the RICs were integrated and compared to those of DPPC in the vesicles-only control incubations, which corresponded to a known amount of lipid. The results are expressed as picomoles of oxidized lipid produced per 10^6 cells in the incubation, and correspond to the mean and standard error of the data from four separate experiments.

With all the oxidized products detected, significant amounts were only found in the incubations containing both phagocytes and phospholipid vesicles; the levels in the vesicles-only and cell-only controls were very low, mostly undetectable. This supports the conclusion that the oxidized products were generated by cellular oxidation during the incubation period, rather than in the subsequent extraction procedures, as these were the same for all the incubations. The percentage conversion of native PAPC to oxidized products was very low, as the signal intensity of the oxidized products was only 0.5–5% of the native PAPC signal in the control treatments.

DISCUSSION

The aim of this study was to investigate the oxidation of lipids by activated phagocytes using a technique which would allow the simultaneous observation of a variety of lipid oxidation products with comparable sensitivity. In particular, we were interested in identifying specific products of phospholipid oxidation that could be of relevance to atherosclerosis. We used HPLC coupled to electrospray mass spectrometry to monitor the phospholipid oxidation, as it is well established that this is an excellent method for detecting and identifying a range of different oxidation products.[13,14,17] Phospholipid vesicles were incubated with cell preparations, in the presence or absence of the stimulant PMA, and controls without either cells or vesicles were also carried out to ensure that both components were required for the production of oxidized species. It was noted that the phospholipid vesicles alone were able to stimulate some release of superoxide by the phagocytes, as observed both by the reduction of NBT, and the appearance of several oxidation products of phosphatidylcholine. This is in agreement with other studies that have reported that certain mixtures of phosphatidylcholines increase phagocyte activity and ROS production.[18] The mechanism by which liposomes or vesicles induce the stimulation of phagocytes is unclear, although membrane perturbation seems a possible candidate.

The data clearly showed the formation of monohydroperoxides and bis-hydroperoxides of PAPC, the polyunsaturated component of the vesicles, following treatment with PMN preparations. However, the most intense oxidation product appeared at $828\,m/z$ ($+46\,m/z$), and was identified as 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine, based on fragmentation and chemical modification studies and by comparison with previously published data.[12] Isoprostanes are isomers of prostaglandins that are generated by free radical attack on arachidonic acid, usually non-enzymatically, and are widely regarded as good markers of lipid peroxidation and oxidative stress owing to their stability.[19,20] A large number of different isomers may be formed, although much attention has focused on the F2-isoprostanes, especially 8-isoprostane $F_{2\alpha}$ (formerly 8-iso-$PGF_{2\alpha}$).[21] There is also some evidence that this compound can be produced by phagocytic cells. Alveolar macrophages were found to generate 8-iso-$PGF_{2\alpha}$ during hyperoxia in the presence of free or esterified arachidonic acid, although cyclooxygenase may have been partially responsible for this process.[22] It has also been demonstrated as a product of LDL following incubation with human monocytes activated with a variety of stimuli, again by both free radical or enzyme catalysed pathways.[23] Thus a free isoprostane of different structure and class has been detected previously as a product of phagoctye-mediated oxidation, but not a phospholipid-esterified isoprostane.

This study shows that intact phospholipid oxidation products, as opposed to lipid peroxidation breakdown products, can be detected in phospholipid vesicles following a 4 h incubation with both activated neutrophils and MNCs. This finding is important in view of emerging evidence for the biological and inflammatory activity of oxidized phospholipids.[24] It is particularly exciting that phagocyte oxidation can result in the formation of substantial amounts of 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine, as this compound has been found in fatty streak lesions of cholesterol-fed rabbits, and is known to increase monocyte-endothelial cell binding. Very recently, it has also been shown to induce the synthesis of monocyte chemotactic protein-1 and interleukin-8

by endothelial cells, which could explain the increased adhesion, and it has been found to accumulate in cells treated with interleukin-1β.[25] Our study suggests that another possible source of this compound *in vivo* could be phagocyte-mediated phosphatidylcholine oxidation following triggering of the respiratory burst, for example, by stimuli that signal via activation of protein kinase C. Interestingly, no significant levels of the chain-shortened PAPC oxidation products, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(2-glutaryl)-*sn*-glycero-3-phosphocholine, were observed in the phagocyte oxidized vesicles. This may reflect the occurrence of different oxidation mechanisms in autoxidation compared to phagocyte-induced oxidation; the latter appears to favour the formation of hydroperoxides and their rearrangement products. The free radical mechanism of attack is probably the result of generation of reactive oxygen species by the NADPH oxidase, rather than an enzymatic process catalysed, for example by 15-lipoxygenase, but further studies are required to confirm this.

Overall, this study strengthens the hypothesis that specific phospholipid oxidation products with atherogenic activity may be produced by phagocytes present in atherosclerotic lesions. Although both PMNs and MNCs were found to produce these compounds, the response of the MNCs may be of more importance to atherosclerosis, as oxidation of LDL is considered more likely to occur within the plaque than in the plasma,[5] and monocytes or macrophages are thought to outnumber other immune cells within such lesions. On the other hand, it has been demonstrated that neutrophils do infiltrate atherosclerotic lesions,[26] and it has also been shown that neutrophils in inflammatory loci can have an extremely short half life (as short as 20 min), in contrast to macrophages which survive much longer and phagocytose other cells.[27] Thus the role of oxidized phospholipid production by neutrophils may also be relevant to atherosclerosis.

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