

Fatty acid and phospholipid chlorohydrins cause cell stress and endothelial adhesion[★]

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The oxidation of low-density lipoprotein (LDL) is thought to contribute to atherogenesis, which is an inflammatory disease involving activation of phagocytic cells. Myeloperoxidase, an enzyme which is able to produce hypochlorous acid (HOCl), is released from these phagocytic cells, and has been found in an active form in atherosclerotic plaques. HOCl can oxidize both the lipid and protein moiety of LDL, and HOCl-modified LDL has been found to be pro-inflammatory, although it is not known which component is responsible for this effect. As HOCl can oxidize lipids to give chlorohydrins, we hypothesized that phospholipid chlorohydrins might have toxic and pro-inflammatory effects. We have formed chlorohydrins from fatty acids (oleic, linoleic and arachidonic acids) and from phospholipids (stearoyl-oleoyl phosphatidylcholine, stearoyl-linoleoyl phosphatidylcholine and stearoyl-arachidonoyl phosphatidylcholine), and investigated various biological effects of these oxidation products. Fatty acid and phospholipid chlorohydrins were found to deplete ATP levels in U937 cells in a concentration-dependent manner, with significant effects observed at concentrations of 25 μ M and above. Low concentrations (25 μ M) of stearoyl-oleoyl phosphatidylcholine and stearoyl-arachidonoyl phosphatidylcholine chlorohydrins were also found to increase caspase-3 activity. Finally, stearoyl-oleoyl phosphatidylcholine chlorohydrin increased leukocyte adhesion to artery segments isolated from C57Bl/6 mice. These results demonstrate potentially harmful effects of lipid chlorohydrins, and suggest that they may contribute to some of the pro-inflammatory effects that HOCl-modified low density lipoprotein has been found to induce.

Keywords: chlorinated phospholipids, chlorinated fatty acids, ATP depletion, atherosclerosis, leukocyte adhesion

INTRODUCTION

Atherosclerosis is now considered as an inflammatory condition, involving immune cell activation and infiltration into the intima, together with increased infiltration of low density lipoprotein (Ross, 1993; Madamanchi *et al.*, 2005). It is also well established that the oxidation of low density lipoprotein (LDL) is a key risk factor for atherosclerosis (Witztum & Steinberg, 1991), and that oxidized LDL (oxLDL) can contribute to the disease pathology *via* several different mechanisms. One way that LDL

can be oxidized is by the action of the phagocyte enzyme myeloperoxidase, which produces HOCl, among other oxidants. There is now very strong evidence for the involvement of myeloperoxidase in atherosclerosis (Podrez *et al.*, 2000; Brennan & Hazen, 2003). Early research showed that myeloperoxidase is present in an active form in atherosclerotic lesions (Daugherty *et al.*, 1994), and HOCl-modified epitopes of LDL were detected in lesions using a specific monoclonal antibody (Hazell & Heinecke, 1997). It was also found that chlorotyrosine derivatives, which are considered to be markers of mye-

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Abbreviations: AA, arachidonic acid; HOCl, hypochlorous acid; LA, linoleic acid; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; OA, oleic acid; PMSF, phenylmethylsulfonyl fluoride; SAPC, stearoyl-arachidonoyl phosphatidylcholine; SLPC, stearoyl-linoleoyl phosphatidylcholine; SOPC, stearoyl-oleoyl phosphatidylcholine.

loperoxidase activity, have been detected at various stages of lesion development and are elevated in LDL and HDL isolated from atherosclerotic lesions (Hazen & Heinecke, 1997; Bergt *et al.*, 2004). Chlorinated lipids, in the form of α -chloro fatty aldehydes, have also been detected in atherosclerotic plaque samples (Thukkani *et al.*, 2003). As well as products of myeloperoxidase and HOCl attack, the free HOCl concentration may reach high concentrations in inflammatory sites, with one study estimating concentrations in the region of 300 μ M (Katrantzis *et al.*, 1991).

HOCl is an oxidizing and chlorinating compound that is able to attack many biological molecules. When incubated with low density lipoprotein, it causes both protein oxidation, of which lysine and cysteine residues react particularly readily, and lipid oxidation and peroxidation (Hazell *et al.*, 1997; Carr *et al.*, 2000). The main reaction of HOCl with unsaturated fatty acyl chains is the formation of chlorohydrins (Winterbourn *et al.*, 1992), and it is known that treatment of isolated human LDL with either HOCl or myeloperoxidase, at concentrations similar to those thought to occur in atherosclerotic lesions, results in generation of phospholipid chlorohydrins (Jerlich *et al.*, 2000). It has been found that HOCl-oxidized LDL has some pro-inflammatory effects, such as induction of the chemotactic cytokine IL-8 in human monocytes and stimulation of neutrophil chemotaxis (Woenckhaus *et al.*, 1998), activation of the NADPH oxidase in macrophages derived from THP-1 and U937 cells (Nguyen-Khoa *et al.*, 1999), and enhanced adhesion of polymorphonuclear leukocytes to human umbilical vein endothelial cells (Kopprasch *et al.*, 1998). For some effects of HOCl-modified LDL, it has been suggested that they are mediated *via* oxidation of the protein moiety (Nguyen-Khoa *et al.*, 1999), but there is still considerable doubt for other effects, and overall the precise modifications and mechanisms responsible have not been identified.

As it is known that free radical oxidized phospholipids have a variety of pro-inflammatory effects (Watson *et al.*, 1997; Subbanagounder *et al.*, 2002a; Spickett & Dever, 2005), and lipid chlorohydrins have previously been found to cause cell lysis, toxicity, and loss of muscle tension (Carr *et al.*, 1997; Iwase *et al.*, 1997; Vissers *et al.*, 2001), we hypothesized that phospholipids and fatty acid chlorohydrins could contribute to deleterious and pro-inflammatory effects of HOCl-modified LDL. In a previous study we had found that phospholipid chlorohydrins caused toxicity to HL60 cells similar to that observed following treatment with autooxidized palmitoyl-arachidonoyl phosphatidylcholine and the lipid peroxidation product 4-hydroxynonenal (Dever *et al.*, 2003). Here we have extended the study

to look at the effects of fatty acid chlorohydrins and phospholipid chlorohydrins on U937 cell ATP levels and caspase-3 activation, and have investigated whether phospholipid chlorohydrins can induce endothelial-leukocyte adhesion, which is an early event in atherosclerotic plaque formation.

MATERIALS AND METHODS

C57Bl/6 (Harlan, UK Ltd) mice were bred in-house at the University of Strathclyde under a project licence authorised under the UK Animals (Scientific Procedures) Act. All phospholipids and fatty acids were purchased from Sigma-Aldrich Chemical Company (Poole, UK), and were prepared individually in chloroform at a concentration of 10 mg/mL. The myeloid cell line U937 was obtained from the European Collection of Animal Cell Cultures (UK).

Preparation of chlorohydrins. Aliquots of each lipid were dried under nitrogen and reconstituted by whirlmixing in an equal volume of Hanks buffered saline solution (HBSS), followed by sonication at 35°C for 15 min, to form lipid vesicles. The vesicles were treated with a 10-fold molar excess of sodium hypochlorite at pH 6.0 until optimal conversion of the native lipid was achieved (between 15 and 50 min, depending on the lipid used). Excess hypochlorite was removed by passing the incubation mixture through reverse phase Sep-pak cartridges (Waters, UK) for phospholipids or normal phase cartridges for fatty acids. The columns were washed with water followed by methanol, and lipids were eluted with 1:1 methanol/chloroform followed by 100% chloroform. The organic solvent was removed under a stream of nitrogen and the lipid chlorohydrins were reconstituted in HBSS and filtered through a 0.2 micron gamma-sterilised filter prior to addition to the cells.

Analysis of chlorohydrins. The conversion of native lipids to chlorohydrins was monitored by electrospray mass spectrometry (ES-MS). One microlitre aliquots were removed and diluted 200-fold with 71:5:7 (by vol.) methanol/hexane/0.1 M ammonium acetate. ES-MS was carried out on Platform (VG) or LCQ-Duo (Finnigan) mass spectrometers in positive ion mode by direct infusion. A spectrum in the range 650–1050 m/z was obtained for phospholipids and 200–600 m/z for fatty acids, and both loss of the native lipids and appearance of chlorohydrins at multiples of +52/+54 m/z was monitored as described previously (Jerlich *et al.*, 2000).

Analysis of cellular ATP levels for viability. U937 cells were cultured at 37°C in RPMI-1640 medium containing 2 mM L-glutamine and 25 mM Hepes, and supplemented with 10% foetal calf serum, 80 U/mL penicillin, and 0.08 mg/mL strepto-

mycin. Treatments were carried out in 96-well luminescence plates (Greiner, UK) containing 1×10^4 cells per well (100 μ L volume). Aliquots (100 μ L) of the sterile chlorohydrin preparations were added to wells, and the plate was incubated at 37°C in a humidified atmosphere with 95% O₂ and 5% CO₂ for 24 h. At the end of this period, the cellular ATP levels were determined by the CellTitre-Glo™ Luminescence assay (Promega), as described previously (Dever *et al.*, 2003). The results are expressed as a percentage of control viability, to allow comparison between individual experiments.

Caspase-3 assays. Caspase-3 activity was measured by a colorimetric assay based on the hydrolysis of acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (Ac-DEVD-pNA) to release *p*-nitroaniline (pNA). U937 cells (2×10^6 cells in 2.5 mL) were treated with phospholipid chlorohydrins. Following treatment, cells were washed with phosphate buffered saline (PBS) and then disrupted in 100 μ L of lysis buffer (50 mM Hepes, pH 7.5, 0.1% Chaps, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, 0.5 mM PMSF, 1 μ g/mL aprotinin, 10 μ M leupeptin, 1 μ M pepstatin) by 15 min incubation on ice, 15 min sonication, and freeze-thawing. The cellular extract was clarified by centrifugation at $10000 \times g$ at 4°C and 25 μ L were used per assay in 96-well plates. Ac-DEVD-pNA was prepared in assay buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 μ g/mL aprotinin, 10 μ M leupeptin, 1 μ M pepstatin) and used at a final concentration of 200 μ M in a total assay volume of 200 μ L/well. Selected wells containing cell extract were also pre-treated with the specific caspase-3 inhibitor Ac-DEVD-CHO for 30 min at 10 μ M.

Leukocyte isolation and radiolabelling. C57Bl/6 (18–24 g) mice were euthanized by CO₂ asphyxiation. Spleens were removed and disrupted over a 200 μ m mesh (Cadisch Ltd) into 3 mL of RPMI-1640 medium (Dutch Modification: Invitrogen Ltd, UK) containing 10% foetal calf serum (Invitrogen). The resulting cell suspensions were passed through a 200 μ m mesh and centrifuged at 125 g for 10 min. The supernatants were removed, and the pellets containing the cells were agitated with 4 mL of distilled water for 30 s to lyse erythrocytes; 4 mL of 1.8% NaCl were then added, to maintain an isotonic solution, and the leukocyte suspensions were passed through a 200 μ m mesh and centrifuged. The resulting cell pellets were re-suspended to a final density of 1×10^6 cells per mL. One millilitre of the re-suspended leukocytes was labelled for 1 h at 37°C in a humidified chamber with 5 μ L (185 kBq) of ⁵¹Cr (as Na₂CrO₄; Amersham Biosciences, UK). The cells were washed twice with RPMI and re-suspended in RPMI to 1×10^6 cells per mL.

Artery segment preparation and leukocyte adhesion. Homologous aortic trees from the euthanized C57Bl/6 mice were removed and cut into three segments (aortic arch, thoracic aorta and abdominal aorta). Each segment was pinned out luminal-side up onto a Sylgard block (Dow Corning, Germany) before placing in a humidified chamber at 37°C, and incubating with RPMI-medium or 25 μ M SOPC chlorohydrin for 1, 2 or 4 h. The segments were washed with RPMI before addition of 5 μ L aliquots of ⁵¹Cr-labelled cells to the artery segments and incubation for 30 min. The segments were then washed again with RPMI, transferred into microtubes and assayed for ⁵¹Cr on a gamma counter (Cobra™ Auto-gamma®, Packard, Canberra Company, UK). Microtubes containing 5 μ L aliquots of labelled and unlabelled cells were also included in the counting process, to allow calculation of leukocyte adhesion as a percentage of the total radioactivity added to the tissue segments.

Following the adhesion assay, tissue samples were fixed in 4% formaldehyde in saline, washed in PBS before dehydration through graded alcohols and HistoClear (Fisher, UK), and embedded in paraffin wax. Sections (4 microns) were cut and mounted on glass slides for haematoxylin and eosin staining.

Statistical analysis. Multiple group comparisons for percentage splenocyte adhesion were performed using One-way ANOVA and Tukey's Post-Hoc test. For analysis of data from caspase-3 assays and ATP levels, a Student's *t*-test was used to compare native *versus* chlorohydrin treatments, and One-way ANOVA was used for comparison of treatment concentrations *versus* control (0 μ M lipid or chlorohydrin).

RESULTS

The formation of chlorohydrins of the phospholipids and fatty acids was monitored using direct infusion electrospray mass spectrometry in positive ion mode. The spectra of the chlorohydrin preparations of stearyl-oleoyl phosphatidylcholine (SOPC), stearyl-linoleoyl phosphatidylcholine (SLPC) and stearyl-arachidonoyl phosphatidylcholine (SAPC) were essentially as reported previously (Dever *et al.*, 2003), and showed nearly complete conversion of the native lipid to the chlorohydrin for SOPC and SLPC, and approx. 50% conversion for SAPC (not shown). Likewise with the fatty acid chlorohydrins, it was found that oleic acid had undergone almost complete conversion to the chlorohydrin form. Linoleic acid had undergone approx. 80% conversion to chlorohydrins with the mono-chlorohydrin predominating but some bis-chlorohydrin also present. With arachidonic acid, as with SAPC, a less complete

conversion of approx. 60% to multiple chlorohydrin forms was achieved. More extensive conversion was not attempted, in order to avoid the formation of lysolipids, which has been reported previously for the arachidonate moiety (Panasenko *et al.*, 2003). The mono-chlorohydrin of arachidonic acid was the major product, but bis-, tris-, and tetra-chlorohydrins

with various mixtures of ^{35}Cl and ^{37}Cl isotope ratios were also found (not shown).

Cultured myeloid cells were incubated with both native lipids and the chlorohydrin preparations for 24 h, and the cellular ATP levels were then determined (Fig. 1). Panels A, C, and E show the effect of the phospholipid chlorohydrins on U937 cells, while

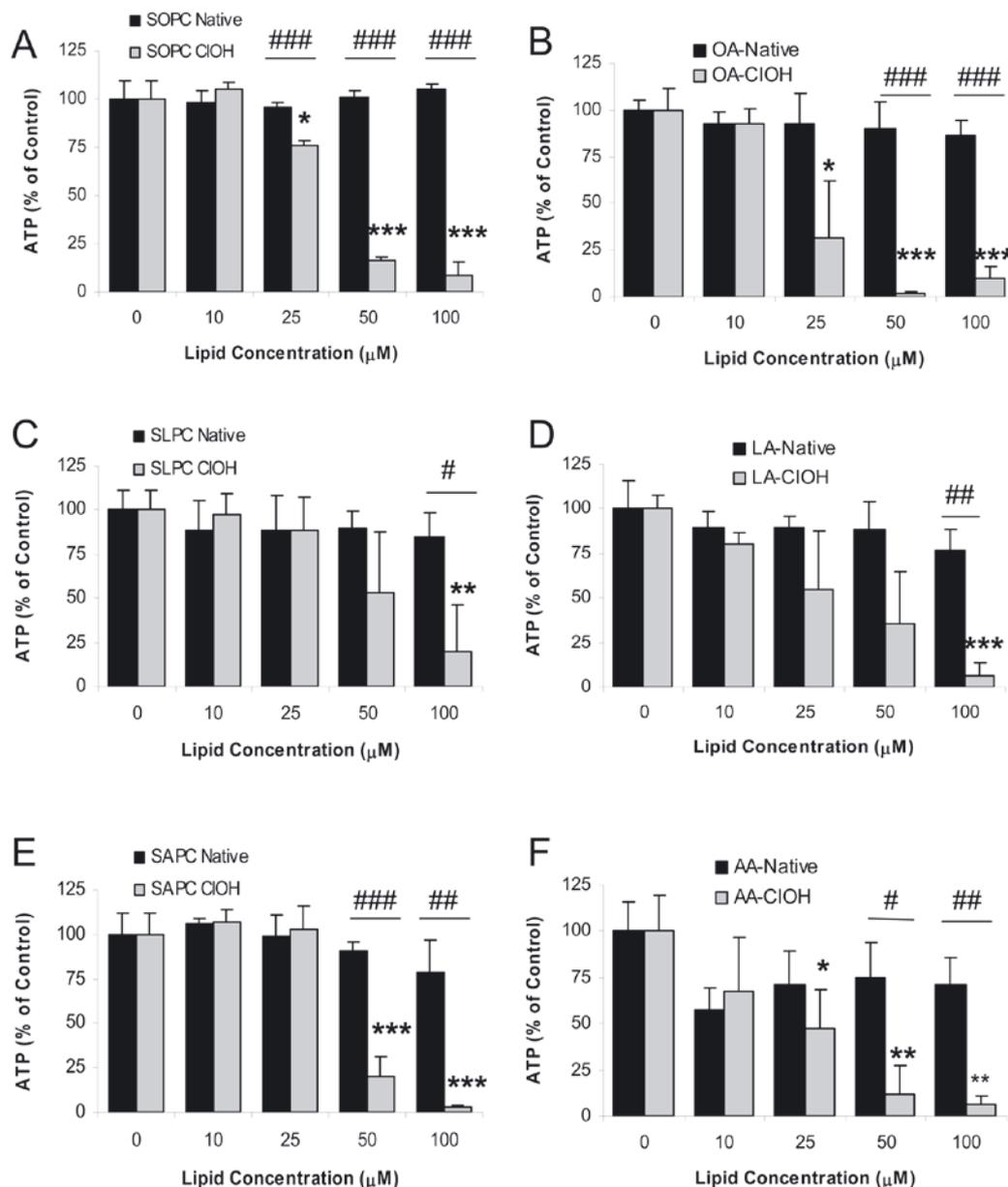


Figure 1. Effect of fatty acid and phospholipid chlorohydrins on ATP levels.

U937 cells were incubated for 24 h in RPMI-1640 medium containing chlorohydrins at the concentrations indicated in the graphs. Following treatment, the cellular ATP level was determined using luciferin-luciferase in a bioluminescent assay, and the percentage ATP level of the cells was calculated by comparison with untreated cells. In each panel the dark bars show treatment with native lipid, and light bars show treatment with the corresponding chlorohydrin. A) Stearoyl-oleoyl phosphatidylcholine; B) oleic acid; C) stearoyl-linoleoyl phosphatidylcholine; D) linoleic acid; E) stearoyl-arachidonoyl phosphatidylcholine; F) arachidonic acid. The data are the average of 3 separate experiments each carried out in triplicate, and the error bars correspond to 1 standard deviation. Significant differences compared to control (0 mM) are shown by * and differences compared to native lipid are shown by #, where 1 symbol corresponds to $P < 0.05$, 2 symbols corresponds to $P < 0.01$, and 3 symbols corresponds to $P < 0.001$.

panels B, D, and F show the effect of the fatty acid chlorohydrins on these cells. It can be seen that all of the lipid chlorohydrin treatments caused depletion of cellular ATP compared to control cells incubated with RPMI medium only, and also compared to cells treated with an equivalent concentration of unmodified lipid. It appeared that the free fatty acid chlorohydrins caused slightly greater depletion of ATP than the corresponding phospholipid chlorohydrins: this was significant for oleic acid *versus* SOPC at 25 μM ($P = 0.004$) and 50 μM ($P = 0.01$), but there were no significant differences for linoleic acid *versus* SLPC or arachidonic acid *versus* SACP, though the latter just failed to reach significance at 25 μM ($P = 0.09$). Both the free form and esterified forms of oleic acid appeared to cause the greatest ATP depletion, followed by the free and esterified forms of arachidonic acid, with linoleic acid in either form having the least effect on the cellular ATP. However, it should be noted that the conversion of SACP and

arachidonic acid to chlorohydrins was not as complete as the other two lipids, and consequently the real effects of SACP chlorohydrin and arachidonic acid chlorohydrin are slightly greater than is apparent from the graph. It was noted that treatment with unmodified SACP also caused a significant loss of ATP, in agreement with previous observations (Dever *et al.*, 2003); this effect was less pronounced with free arachidonic acid.

To investigate whether apoptosis might contribute to the toxicity observed with the chlorohydrins, caspase-3 activation was monitored in U937 cells incubated with SOPC chlorohydrin or SACP chlorohydrin for 24 h (Fig. 2). It was found with both lipids that incubation with 25 μM chlorohydrin, the lowest concentration tested in this assay, caused a significant increase in caspase-3 activity (approx. 100–150% increase). Increasing the chlorohydrin concentration further resulted in a decrease in caspase-3 activity compared to 25 μM . Inclusion of the specific caspase-3 inhibitor Ac-DEVD-CHO in the assay resulted in a substantial abrogation of caspase-3 activation.

SOPC chlorohydrin was then selected for further studies of more physiological effects of chlorohydrin using an *ex-vivo* model of leukocyte-arterial adhesion as described previously (Lim *et al.*, 2006; Dever *et al.*, 2006). Adhesion of splenocytes to the endothelial surface could be visualized by micrographs (Fig. 3A) and was quantitated by ^{51}Cr -labelled splenocyte-dependent radioactivity of the arterial segments. It was found that incubation of mouse artery segments with SOPC chlorohydrin caused significant enhancement of splenocyte adhesion to the endothelium (Fig. 3B). Incubation of artery segments with 25 μM SOPC chlorohydrin for 10 min was not long enough to stimulate increased adhesion of radiolabelled splenocytes, but longer incubation times (1–4 h) caused approximately a 3-fold increase in adhesion of splenocytes to aortic, thoracic and abdominal artery segments. The increase in all segments was maximal or nearly maximal at 1 h; extending the incubation period to 2 or 4 h did not greatly enhance the splenocyte adhesion. Incubation with unmodified SOPC did not cause any increase in adhesion (not shown).

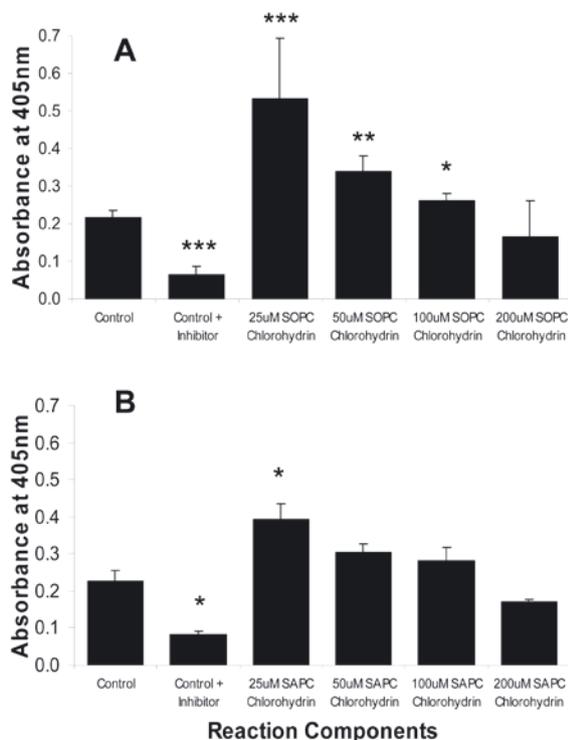


Figure 2. Effect of phospholipid chlorohydrins on caspase-3 activity.

U937 cells were incubated for 24 h in medium containing the chlorohydrins. The caspase-3 activity was assayed in cell extracts by measuring absorbance at 405 nm corresponding to the release of *p*-nitroaniline from acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (Ac-DEVD-pNA). Pre-treatment with the specific caspase-3 inhibitor Ac-DEVD-CHO was for 30 min at 10 μM . Controls correspond to cells treated with medium only. A) Stearoyl-oleoyl phosphatidylcholine chlorohydrin ($n = 4$), and B) stearoyl-arachidonoyl phosphatidylcholine chlorohydrin ($n = 2$). Error bars correspond to 1 standard deviation. Significant differences are relative to control (0 mM), where * corresponds to $P < 0.05$, ** to $P < 0.01$, and *** to $P < 0.001$.

DISCUSSION

It is becoming increasingly accepted that HOCl-modified LDL is important in atherosclerosis, but the contributions of lipid modifications *versus* protein modifications are not completely understood. As HOCl treatment of LDL generates chlorohydrins, these could contribute to adverse effects of HOCl-LDL at a cellular and tissue level. The first finding

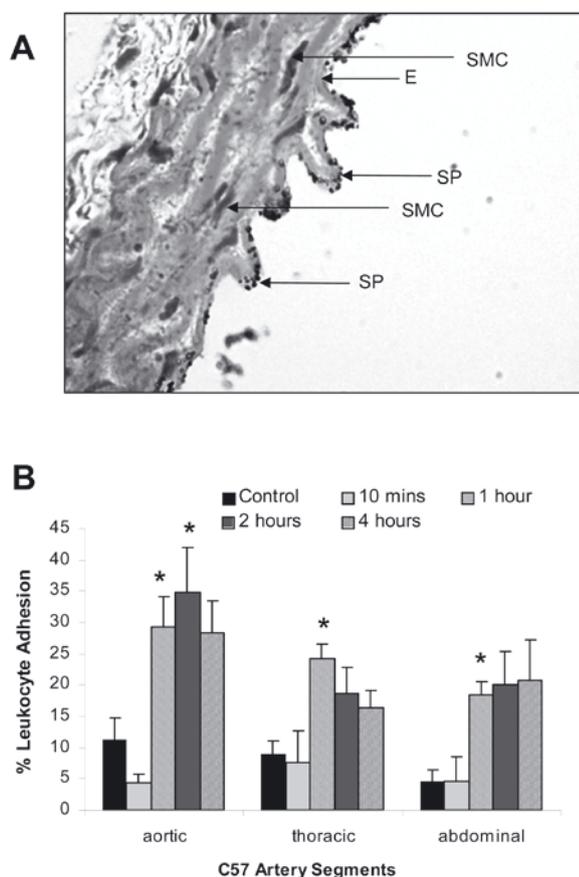


Figure 3. Effect of SOPC chlorohydrin on splenocyte-arterial adhesion.

C57Bl/6 artery segments were incubated with 25 μ M stearoyl-oleoyl phosphatidylcholine chlorohydrin before addition of ^{51}Cr -labelled splenocytes for 30 min. A) Micrograph of an artery segment stained with haematoxylin and eosin showing adherent splenocytes (SP), endothelium (E), and smooth muscle cells (SMC). B) The adhesion of splenocytes was quantitated by measurement of segment radioactivity and the results are shown as percentage of the total radioactivity added per segment. The segments were incubated with chlorohydrin for 0 min (control, $n = 11$), 10 min ($n = 5$), 1 h ($n = 10$), 2 h ($n = 8$) or 4 h ($n = 10$). Error bars are 1 standard error of the mean and *indicates $P < 0.05$ compared to control.

of this study was that both phospholipid and fatty acid chlorohydrins caused similar depletion of cellular ATP levels in cultured myeloid U937 cells. The loss of cellular ATP indicates that the cells are stressed and that loss of viability is occurring, which is in agreement with previous studies that have shown toxic effects of fatty acid or cholesterol halo-hydrins on erythrocytes and endothelial cells (Carr *et al.*, 1997; Vissers *et al.*, 2001). However, measurement of cellular ATP levels cannot distinguish between apoptosis and necrosis, so the effect of SOPC- and SAPC-chlorohydrins on cellular caspase-3 activity was also assayed to investigate whether a lipid chlorohydrin might be able to induce apoptosis. Caspase-3 activation was evident at lower treat-

ment severity than loss of ATP and diminished as the treatment concentration increased, consistent with the idea that the mode of cell death is dose-dependent, with lower doses causing apoptosis and higher doses diminishing apoptosis but increasing necrosis (Buttke & Sandstrom, 1994; Vayssier *et al.*, 1998; Vissers *et al.*, 1999). Similar concentrations (10 μ M) of palmitoyl-arachidonoyl phosphatidylcholine oxidation products have previously been found to induce apoptosis and caspase-3 activation in arterial smooth muscle cells (Loidl *et al.*, 2003), *via* activation of acid sphingomyelinase. Possibly the chlorinated oxidation products of phospholipids could act by a similar mechanism.

Our results suggest that lipid chlorohydrins may have more complex cellular effects than previously thought. Earlier studies have mainly focused on membrane disruption as a mechanism for toxicity; as chlorohydrins are more polar than native lipids, their formation within membrane or subsequent incorporation was thought to disrupt the lipid bilayer structure, and result in toxicity simply through physical effects (Carr *et al.*, 1997). In studies on human endothelial cells, the mechanism of cell death was described as necrotic rather than apoptotic, and similar to that observed with higher doses of direct HOCl treatment (Vissers *et al.*, 2001), although lower doses of HOCl induced apoptosis (Vissers *et al.*, 1999). However, this may relate to the lipid chlorohydrin concentration used by Vissers *et al.* (2001) when observing morphological changes, of which the lowest tested was 37.5 μ M.

The other major finding of the study was that treatment of arterial segments with SOPC chlorohydrin induced a significant increase in splenocyte adhesion within one hour. The treatment of arterial segments was carried out at a low chlorohydrin concentration (25 μ M) and over relatively short time periods, as previously we have found that toxic effects of phospholipid chlorohydrins only became apparent after 6 h (Dever *et al.*, 2003). This was important to ensure that the increased adhesion observed following chlorohydrin treatment was not simply due to cell death and release of cell components that could be pro-inflammatory. Thus it appears that phospholipid chlorohydrin can cause a pro-inflammatory effect comparable to that observed with autoxidized palmitoyl-arachidonoyl phosphatidylcholine (PAPC) (Watson *et al.*, 1997). Furthermore, the concentration that induces this effect is similar to the concentrations of PAPC oxidation products, such as palmitoyl-oxovaleroyl phosphatidylcholine and hydroxy alkenal derivatives of phosphatidylcholines, which have also been found to cause adhesion of monocytes to human aortic endothelial monolayers, and increased production of MCP-1 and IL-8 (Subbanagounder *et al.*, 2002b). This further supports the

hypothesis that chlorohydrins, in addition to causing necrosis at high concentrations, may act on cells in more subtle ways to alter cell functions. The mechanism of chlorohydrin-induced adhesion to arterial segments is currently unknown, and further study is warranted to investigate this.

This study has presented the first evidence that phospholipid chlorohydrins can induce inflammatory cell-endothelial adhesion, and therefore that

HOCl-modification of the phospholipid moiety of LDL may contribute to the pro-inflammatory and pro-atherogenic effects of HOCl-LDL that have been reported previously.

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