

Fragmentation of extracellular matrix by hypochlorous acid

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The interaction of extracellular matrix with cells regulates their adhesion, migration and proliferation, and it is believed that damage to vascular matrix components is a factor in the development of atherosclerosis. Evidence has been provided for a role for the haem enzyme MPO (myeloperoxidase), released by activated monocytes (and possibly macrophages), in oxidative events within the artery wall. As MPO is released extracellularly, and is highly basic, it might be expected to associate with poly-anionic matrix components thereby localizing damage to these materials. In this study the reaction of the MPO-derived oxidant hypochlorous acid (HOCl) with extracellular matrix from vascular smooth muscle cells and healthy pig arteries has been examined. HOCl is rapidly consumed by such matrix samples, with the formation of matrix-derived chloramines or chloramides. The yield of these intermediates increases with HOCl dose. These materials undergo a time- and temperature-dependent decay, which parallels the re-

lease of sugar and protein components from the treated matrix, consistent with these species being important intermediates. Matrix damage is enhanced by species that increase chloramine/chloramide decomposition, with copper and iron ions being effective catalysts, and decreased by compounds which scavenge chloramines/chloramides, or species derived from them. The effect of such matrix modifications on cellular behaviour is poorly understood, though it is known that changes in matrix materials can have profound effects on cell adhesion, proliferation, growth and phenotype. The observed matrix modifications reported here may therefore modulate cellular behaviour in diseases such as atherosclerosis where MPO-derived oxidants are generated.

Key words: chloramine, extracellular matrix, glycosaminoglycan, hypochlorite, myeloperoxidase, protein oxidation.

INTRODUCTION

The ECM (extracellular matrix) is a complex structure consisting of proteins and proteoglycans that acts as a scaffold for cells in mammalian tissues. The interaction of vascular ECM with surrounding cells regulates their adhesion, migration and proliferation throughout the development of the vasculature and in disease [1]. It is believed that damage or alteration to components of the ECM of the vascular wall is a factor in the development of atherosclerosis. Human atherosclerotic plaques have been reported to contain structural modification of the internal elastic lamina of the artery wall, with the accumulation of lipids reported to occur at such sites [2,3]. In addition, ECM components including elastin and proteoglycans are believed to undergo fragmentation and/or physicochemical alteration during atherogenesis [2,4,5]. An important mechanism that is believed to mediate such damage, as well as other purportedly atherogenic events, is oxidation [6]. Early-through to advanced-stage human atherosclerotic plaques have been reported to contain oxidized lipids, sterols and proteins at significantly higher levels than control tissue (reviewed in [7,8]), together with decreased (though not depleted) levels of antioxidants [9].

Elastin, an important component of the ECM of elastic tissues such as arteries, has been isolated from atherosclerotic plaques and reported to contain fluorescent protein-derived species [10]. This has been ascribed to phenolic cross-links, such as di-Tyr, arising from the oxidation of Tyr residues [11]. Physicochemical changes to ECM materials are also known to affect endothelial cell adhesion [12,13] and smooth muscle cell migration and proliferation [14], processes that are implicated in atherogenesis.

Chemical analysis of proteins isolated from human atherosclerotic plaques has revealed the presence of a wide spectrum of oxidized amino acid side chains which have been interpreted

in terms of the occurrence of two oxidative mechanisms. The first of these involves oxygen-radical formation catalysed by trace transition metal ions; the second involves chlorinating and oxidizing species, such as hypochlorous acid (HOCl) or Cl₂, generated by the haem enzyme MPO (myeloperoxidase) [15–17]. Hypochlorous acid exists as a mixture of HOCl and its anion ⁻OCl at physiological pH values; HOCl will be used hereon to indicate this mixture. MPO is released from activated leucocytes and catalyses the formation of HOCl from physiological concentrations of chloride ions and H₂O₂ (generated via the oxidative burst of leucocytes as well as other cells) [18]. The evolutionary function of HOCl production is bactericidal [19] and it is believed to be the major oxidant produced by neutrophils *in vivo* [18]. However, localized excess production of HOCl has been implicated in the progress of diseases such as atherosclerosis, which can involve an inflammatory or immunological response [20].

It has been shown that both active MPO, and the products of its action, are present in various grades of human atherosclerotic lesions [15,21,22], and that the levels of this enzyme are strongly associated with coronary artery disease [23–25]. MPO is a strongly basic (cationic) protein at physiological pH [18] and binds, via electrostatic interactions, to negatively charged materials such as the polyanionic glycosaminoglycans of the ECM [26] and low-density lipoprotein [27]. Positively charged transition-metal ion complexes would likewise be expected to associate with matrix materials. Such binding might be expected to make the material to which the oxidizing system is bound a major site of damage, and this has been confirmed in some cases [26,28,29]. Thus both MPO, and some metal ion complexes, can oxidize materials to which they are bound in a site-specific manner [30,31]. Evidence has been obtained for the modification of ECM components by MPO-derived oxidants in human atherosclerotic

Abbreviations used: ECM, extracellular matrix; MPO, myeloperoxidase; TNB, 5-thio-2-nitrobenzoic acid; VSMC, vascular smooth muscle cells.

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lesions by use of a monoclonal antibody (HOP-1) raised against HOCl-damaged low-density-lipoproteins. This antibody does not recognize proteins oxidized by a wide variety of other oxidants *in vitro* [32]. Application of this antibody resulted in significant staining of both intra- and extracellular locations, particularly in the intimal area and at cholesterol clefts, with little staining detected in normal human artery samples [32]. Recent studies have demonstrated that the intensity of staining induced by this antibody correlates with intimal thickening in lesions of different severity, suggesting that the MPO-induced damage plays a role in lesion development [33].

HOCl reacts with many biological molecules and can cause damage to tissues [34,35]. A number of studies have demonstrated that MPO-derived oxidants such as HOCl can induce relatively specific damage to proteins and polysaccharides by reaction with a limited number of reactive sites. Thus cysteine, methionine, lysine, tyrosine and tryptophan residues of proteins are known to be particularly susceptible to oxidation, whereas with polysaccharides initial damage is localized at amino or amide (N-acetyl) sugars [34,36,37]. Previously we have shown that the majority of the oxidized proteins accumulated in atherosclerotic plaques reside in the ECM [29], and that oxidized products previously attributed to oxygen radical-mediated damage may arise via further reactions of materials such as chloramines/chloramides [RNHCl and R-NCl-C(O)R' species respectively] generated by MPO-catalysed reactions, as these intermediates have been previously shown to give rise to radicals via transition-metal-ion-catalysed reactions [38,39]. Chloramines and chloramides cannot be identified separately by current assays and hence the phrase chloramines/chloramides is used hereafter to indicate the unknown mixture of these species. The reaction of amine groups such as those on lysine side chains with HOCl (to form chloramines) is much faster than that of backbone amides (to give chloramides) [37], but there are likely to be many more amides than amines in ECM due to the presence of both large numbers of protein backbone amide groups and N-acetyl amide functions in polysaccharide components.

In light of these previous reports, we have examined the formation of chloramines and chloramides on ECM [synthesized by VSMC (vascular smooth muscle cells) *in vitro* or extracted from porcine aortae] and examined whether the decay of these species, in the presence or absence of added metal ions and scavengers, gives rise to matrix fragmentation as evidenced by radionuclide, uronic acid and protein release.

EXPERIMENTAL

Materials

All solutions and media were prepared in high-purity, deionized water. SDS, NaOH, 5,5'-dithiobis-(2-nitrobenzoic acid), L-methionine, Dulbecco's PBS and penicillin/streptomycin were purchased from Sigma (St Louis, MO, U.S.A.). D-Glucose and sodium hypochlorite solution were purchased from BDH (Poole, Dorset, U.K.). L-[U-¹⁴C]Proline and D-[1-³H]glucosamine were purchased from Amersham Biosciences (Uppsala, Sweden). Foetal bovine serum was from Gibco-BRL (Grand Island, NY, U.S.A.), supplied by Life Technologies (Auckland, New Zealand). L-Glutamine and sodium pyruvate were purchased from Trace Scientific (Melbourne, Australia). Dulbecco's modified Eagle's medium (DME/Low) was purchased from JRH Biosciences (Lenexa, KS, U.S.A.). NH₄OH was purchased from ICN (Costa Mesa, CA, U.S.A.). Ultima Gold scintillant was purchased from Packard Bioscience (Groningen, The Netherlands). HOCl solutions were prepared immediately prior to use by the dilution

of a concentrated stock solution [approx. 0.5 M in 0.1 M NaOH (BDH, Poole, U.K.) into 0.1 M phosphate buffer (pH 7.4)]. HOCl concentrations were determined from the absorbance of ⁻OCl at 292 nm (pH 12) using a molar absorbance coefficient ϵ of 350 M⁻¹ · cm⁻¹ [40].

Cell culture and preparation of ECM

VSMC (A7r5) were cultured in media (DME/Low) supplemented with 10% (v/v) foetal bovine serum, 3.5 g · l⁻¹ glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 units · ml⁻¹ penicillin and 100 μ g · ml⁻¹ streptomycin (designated DMEM from here on) in humidified 5% CO₂ at 37 °C. Cells were seeded at 4 × 10⁵ per well in six-well Multiwell™ Falcon tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), with 2 ml of DMEM/well, which yielded a confluent layer of cells after 24 h. Cultures were incubated for up to 2 weeks, with the DMEM being replaced every 2 days. For the production of radiolabelled ECM, the DMEM was supplemented with 0.05 μ Ci · ml⁻¹ L-[U-¹⁴C]proline and 0.5 μ Ci · ml⁻¹ D-[1-³H]glucosamine. Cells were removed from the ECM using 50 mM NH₄OH for 20 min and the ECM was washed three times with nanopure water. The ECM was stored at -20 °C until required.

Solubilization of ECM and measurement of radionuclides

Radiolabelled ECM was efficiently removed from the tissue-culture plastic by the addition of 1 ml/well of 5% SDS in 0.1 M NaOH and incubation at 110 °C for 10 min. This method was found to be more efficient than a published method utilizing 2 M NaOH [41] (results not shown). A 200 μ l aliquot of this solubilized matrix was added to 5 ml of scintillant and the radioactivity measured in a Tri-Carb 2100TR liquid scintillation analyser (Packard Bioscience, Meriden, CT, U.S.A.). In experiments measuring the radioactivity released into solution, a 500 μ l aliquot was added to 5 ml of scintillant and the radioactivity determined.

Preparation of arterial ECM and measurement of protein and polysaccharide

Thoracic aortae were obtained from 16-week-old male Large White × Landrace pigs fed a standard commercial diet. Immediately following death the thoracic aorta was removed, placed in Chelex-treated, argon-flushed, PBS containing butylated hydroxytoluene (100 μ M) and 1 mM EDTA and subsequently stored at -80 °C. The ECM was extracted from the intima of these tissues using a previously published method [29]. The protein and polysaccharide content of ECM samples was determined after complete digestion of the insoluble material with 1 M NaOH at 37 °C, overnight. The protein concentration was then determined using the BCA (bicinchoninic acid) assay (Pierce, Rockford, IL, U.S.A.), using BSA as a standard. The polysaccharide content of this material was estimated by measuring the uronic acid content with the carbazole assay [42]. These analyses gave a yield of 0.40 ± 0.01 g of protein and 5.31 ± 0.13 mg of uronic acid/g of dry-weight ECM.

Treatment of ECM samples with oxidants

ECM isolated from VSMC cultures was initially washed with 2 ml of PBS and then incubated at 37 °C with 1 ml of PBS or PBS containing HOCl, for the designated period. For experiments determining the effect of chloramine/chloramide scavengers and metal ions on fragmentation of HOCl-treated ECM, six wells of

ECM were washed with 50 mM phosphate buffer (pH 7.4) and then incubated with HOCl in 50 mM phosphate buffer (pH 7.4) for 5 min. These ECM samples were then washed with nanopure water and subsequently incubated with either nanopure water or water containing 20 nmol of either $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ or FeCl_3 or 2.5 μmol of either methionine or Trolox C for 55 min at 37 °C. The ECM was then incubated with water for two subsequent 2-h incubations (i.e. 60–180 and 180–300 min). For the metal ion experiments, the ECM was incubated with 20 nmol of metal ion in water for the two subsequent incubations.

ECM extracted from the intima of porcine thoracic aortae was incubated with irreversible protease inhibitors (10 mM iodoacetate and 1 mM PMSF) for 15 min in 50 mM phosphate buffer (pH 7.4). The ECM was then washed with buffer and incubated with HOCl in buffer for 5 min at 37 °C. Following removal of the HOCl, the ECM was incubated in phosphate buffer for the remainder of the experiment.

Measurement of chloramines/chloramides

The presence of chloramines/chloramides on ECM materials, and unconsumed HOCl, was measured by their reaction with TNB (5-thio-2-nitrobenzoic acid) as described previously [12]. Following exposure to 1 ml of HOCl solution (or PBS for controls), the ECM-containing wells were washed three times with fresh PBS. To each ECM-containing well (or supernatant aliquot for HOCl levels) was added 1 ml of TNB reagent, and the plates were then incubated in the dark for 15 min. Following the incubation period the TNB solution was removed and the extinction of the TNB reagent solution was measured at 412 nm by using a molar extinction coefficient of $13\,600\text{ M}^{-1} \cdot \text{cm}^{-1}$ [43]. HOCl consumption was confirmed by measuring the chlorination of monochlorodimedon at 290 nm [34].

RESULTS

Synthesis of ^3H - and ^{14}C -labelled ECM by VSMC in culture

Mammalian cells cultured *in vitro* synthesize an extracellular network of polysaccharides and proteins [44]. However, there is a paucity of information concerning whether polysaccharides and proteins are synthesized in a constant ratio during the life of a culture, and thus whether ECM in culture changes significantly with age. To clarify this matter, VSMCs were grown in the presence of L-[U- ^{14}C]proline and D-[1- ^3H]glucosamine (see the Experimental section). Following four separate incubation periods, cells were removed, the ECM solubilized and the incorporated radioactivity measured. Figure 1 shows that the confluent monolayer of cells synthesized ^3H - and ^{14}C -radiolabelled ECM at a rate that was proportional in a linear manner with the length of incubation under the conditions employed. The results also demonstrate that the ratio of incorporated ^3H to ^{14}C in the ECM remained relatively constant over the measured period.

Effect of HOCl on chloramine/chloramide formation and fragmentation of ECM generated by VSMC

The formation of chloramines/chloramides on ECM by HOCl, was investigated by treatment of ECM isolated *in vitro* from VSMCs with HOCl in PBS, or PBS alone. The HOCl remaining in solution, and the concentration of chloramines/chloramides generated on the ECM, were assayed over time (Figure 2). HOCl is rapidly consumed in the presence of ECM, with the greatest rate of consumption occurring in the first 10 min. Chloramine/chloramide formation was observed on the ECM, in a manner which demonstrated HOCl concentration dependence. The

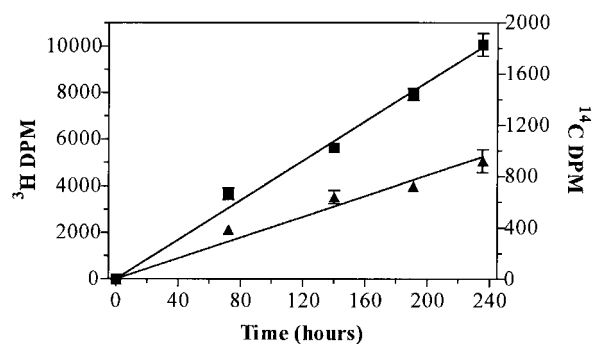


Figure 1 Accumulation of ^3H - and ^{14}C -labelled material in ECM synthesized by VSMC

VSMC were incubated in growth media supplemented with L-[U- ^{14}C]proline and D-[1- ^3H]glucosamine. Following removal of cells with 50 mM NH_4OH , the ECM was solubilized and the radioactivity incorporated into the ECM was measured as described in the Experimental section. Results for ^3H (■) and ^{14}C (▲) are expressed as mean \pm S.D. disintegrations/min (DPM) from three experiments.

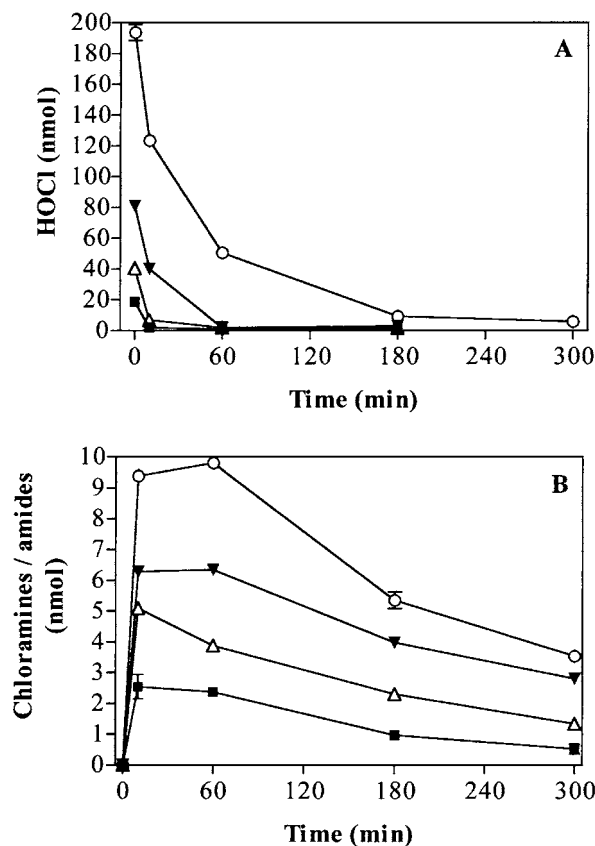


Figure 2 Consumption of HOCl and the formation and decay of chloramines/chloramides during the incubation of ECM with HOCl

ECM synthesized by smooth muscle cells *in vitro* (see the Experimental section) was incubated with 1 ml of PBS containing the following concentrations of HOCl: 200 (○), 80 (▼), 40 (▲) and 20 nmol (■). Residual HOCl in solution and chloramines/chloramides formed on the ECM were measured using the TNB assay as described in the Experimental section. Results for HOCl consumption (A) and chloramine/chloramide formation (B) are expressed following subtraction of control experiments (PBS only), and represent the means \pm S.D. from three replicates; where no error bar is visible it is obscured by the symbol. HOCl consumption data was confirmed at the highest HOCl concentration using the monochlorodimedon assay as described in the Experimental section; both assays gave similar values.

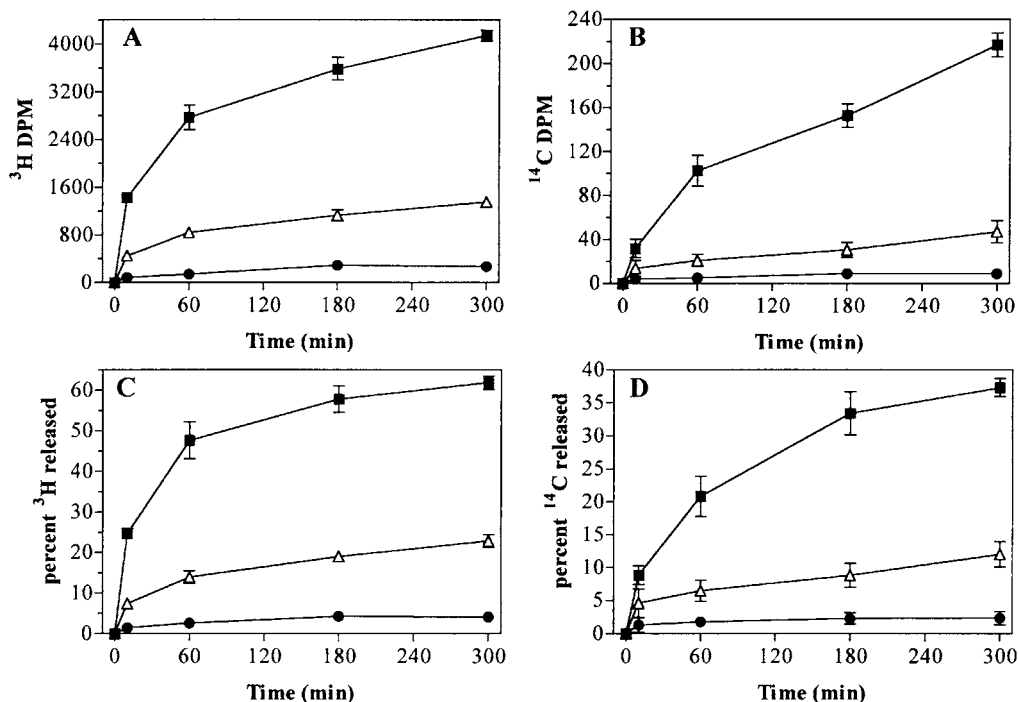


Figure 3 Release of radioactivity from ^3H - and ^{14}C -radiolabelled smooth muscle cell ECM following incubation with HOCl

^3H - and ^{14}C -radiolabelled ECM synthesized by VSMCs *in vitro* (see the Experimental section) was incubated with 1 ml of PBS containing the following concentrations of HOCl: 100 (■), 25 (△) and 0 nmol (●). (A) and (B) show results obtained by measuring the radioactivity released into solution. (C) and (D) show results obtained after solubilizing the ECM to measure the remaining radioactivity and calculating the percentage release. Results are expressed as the means \pm S.D. from six replicates; where no error bar is visible it is obscured by the symbol.

chloramine/chloramide concentration on the ECM reached a maximal level after approx. 10 min of incubation. Significant decay was observed at later time points, with approx. 50% of the chloramines/chloramides lost by 5 h.

The effect of HOCl incubation and chloramine/chloramide decay on the structural integrity of the ECM synthesized by VSMCs was investigated by incubation of the radiolabelled matrix with HOCl, and the released radioactivity measured (Figures 3A and 3B). ECM incubated with HOCl displayed substantial release of radioactivity in the first 10 min, when HOCl consumption is most rapid (Figure 2A). In the absence of HOCl, little release of either ^3H or ^{14}C was detected, indicating that endogenous proteases and glycosaminoglycan-degrading enzymes were either not present or inactive in these preparations. In the HOCl-treated samples, ongoing release of both ^3H and ^{14}C was observed between 10 and 300 min, concomitant with the decay of the chloramines/chloramides (cf. Figure 2B). The level and rate of radioactive release was dependent on the concentration of HOCl employed. To determine the percentage release of each radionuclide, the remaining ECM at each time point was solubilized, and the residual radioactivity measured. The percentage release for each radionuclide is displayed in Figures 3(C) and 3(D). The results demonstrate that treatment with HOCl results in a greater proportional release (approx. 1.7-fold) of ^3H from glucosamine-derived materials than ^{14}C from proline-derived materials from the radiolabelled ECM.

Effect of HOCl concentration and temperature on fragmentation of ECM

Chloramine and chloramide decay has been previously shown to be retarded at low temperatures [38]. To examine the effect of lowering the temperature on fragmentation of ECM containing

chloramines/chloramides, radiolabelled ECM was placed on ice following incubation with HOCl. The radioactivity released from the matrix was then assayed over time (see Figures 4A and 4B). The reduction in temperature resulted in a significant decrease in radioactivity released from the ECM. This reduction in fragmentation was especially pronounced in the 10–60 min period, where there was a 78% reduction in ^3H release and an 86% reduction in ^{14}C release when compared with the results obtained at 37 °C. In the 60–300 min period, the reduction in release of radioactivity was 50% for ^3H and 65% for ^{14}C .

Effect of chloramine/chloramide scavengers and metal ions on fragmentation of HOCl-treated ECM

Methionine and Trolox C were used to determine the effect of removal of chloramines/chloramides (or species derived from these intermediates) on fragmentation of HOCl-treated ECM. Following incubation with HOCl, excess oxidant was removed and the ECM washed. The ECM was then incubated with the scavenger or water for 55 min. These solutions were then replaced with water for two further periods of incubation. Chloramine/chloramide concentrations were measured in analogous experiments using non-radiolabelled ECM. Both methionine and Trolox C significantly reduced chloramine/chloramide levels by the 60 min time point, with methionine being the more effective quenching agent (Figure 5A).

Release of ^3H - and ^{14}C -labelled fragments from the ECM was measured at each time point (Figures 5C and 5D). Both agents effected a significant reduction in both ^3H and ^{14}C released from HOCl-treated ECM. In contrast to the effects of these two agents on the chloramine/chloramide levels, Trolox C had a greater inhibitory effect on radiolabel release. The higher level of radiolabel release observed with methionine may in part be attributed to the

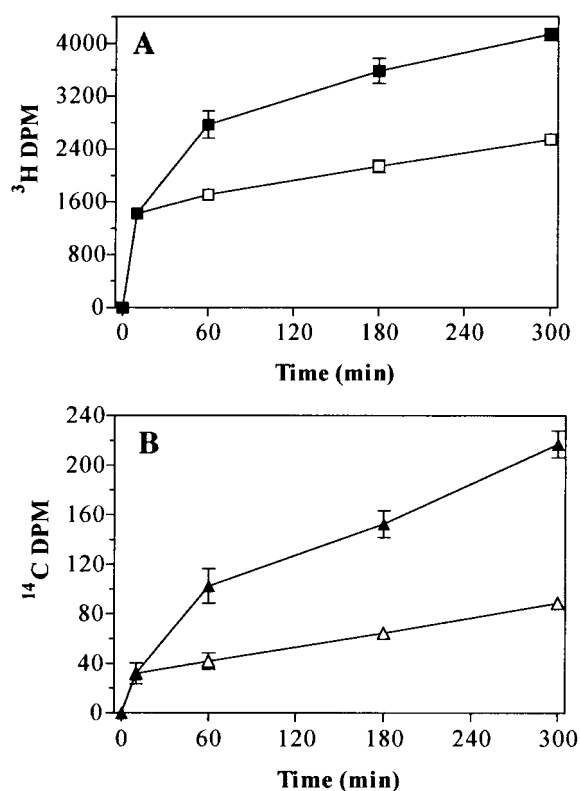


Figure 4 Effect of incubation temperature on ECM fragmentation induced by HOCl

(A) ³H released from radiolabelled smooth muscle cell ECM incubated with 100 nmol HOCl in 1 ml of PBS at 37 °C (■) or 37 °C for 10 min and subsequently at 4 °C (□). (B) ¹⁴C released from radiolabelled ECM incubated with 100 nmol HOCl in 1 ml of PBS at 37 °C (▲) or 37 °C for 10 min and subsequently at 4 °C (△). Results are expressed as the means ± S.D. from six replicates; where no error bar is visible it is obscured by the symbol.

increased release of both ³H- and ¹⁴C-labelled fragments observed in the non-HOCl treated controls treated with methionine when compared with both Trolox C- and non-antioxidant-treated samples (Figures 5C and 5D).

Metal ions are known to catalyse the decay of chloramines and chloramides (e.g. [45]). To determine whether metal ions would catalyse the decay of ECM chloramines/chloramides, and/or cause increased fragmentation of the matrix, radiolabelled ECM was treated with HOCl, washed, then incubated with 20 nmol of Cu²⁺ or Fe³⁺. Chloramine/chloramide concentrations were measured in analogous experiments using non-radiolabelled ECM (Figure 5B). The presence of Cu²⁺ or Fe³⁺ decreased the levels of chloramines/chloramides on the ECM, with the extent of decomposition being greater than that detected in the control (no metal ion) samples. Release of ³H- and ¹⁴C-labelled fragments from the ECM was also measured over time (Figures 5E and 5F). The presence of Cu²⁺ caused a marked increase in the release of ¹⁴C-, but not ³H-, labelled fragments from the HOCl-treated ECM. In contrast, Fe³⁺ caused a significant reduction in the extent of release of ³H-labelled fragments and a less marked reduction in release of ¹⁴C-labelled fragments. In the absence of the HOCl pre-treatment, no effect was observed on radiolabel release from the ECM, except for the effect of Cu²⁺ on ³H-labelled fragments. The observed reduction in release of these fragments in the presence of Cu²⁺ may in part explain the lack of ³H-labelled fragment release, in contrast to increased ¹⁴C-labelled fragments, from HOCl-treated ECM.

In order to determine whether the observed reduction in release in radiolabelled fragments in the presence of Fe³⁺, and the apparent favoured release of ¹⁴C in the presence of Cu²⁺, was due to precipitation of radiolabelled fragments with the metal ion, radiolabelled ECM was incubated with HOCl and then treated with 20 nmol of Cu²⁺ or Fe³⁺, or water alone. Subsequently, the HOCl-treated ECM was incubated with EDTA to chelate the metal ions and release any bound matrix fragments. The presence of EDTA resulted in a significantly greater release of radiolabel from samples that were treated with metal ions than the control (no metal ion) samples, indicating that a proportion of the released material was bound to the metal ions (Figure 6).

Effect of HOCl on ECM extracted from the intima of pig aortae

In order to demonstrate that ECM from mammalian aortae responds similarly to HOCl treatment as that produced *in vitro* by VSMC, ECM extracted from pig aortae was incubated with HOCl, and consumption of this oxidant and formation and decay of chloramines/chloramides assayed as described above (Figures 7A and 7B). Polysaccharide and protein release from the ECM was subsequently determined by measurement of uronic acid and peptide release respectively (see the Experimental section). The results obtained (Figures 7C and 7D) demonstrate that the release of these materials mirrors the consumption of HOCl and the formation and decay of chloramines/chloramides on the ECM. In accord with the results obtained with the radiolabelled ECM synthesized *in vitro*, there was greater proportional release of polysaccharide (measured by the uronic acid assay) than protein from the HOCl-treated ECM.

DISCUSSION

In this study we have demonstrated that ³H-glucosamine and ¹⁴C-proline are incorporated into the ECM by confluent monolayers of VSMC at a constant rate. Thus it would appear that polysaccharides containing ³H-glucosamine, and proteins containing ¹⁴C-proline, are synthesized concurrently by the cells during production of ECM. These data indicate that variability in culture growth between samples does not significantly change the proportion of radioligands incorporated into the ECM, and thus such variability should not impact significantly on any analysis of incorporated radioactivity.

We have demonstrated previously that oxidized proteins are present on the ECM of human atherosclerotic plaques, and that these materials are consistent with HOCl-mediated oxidation [29]. These data are consistent with proteins being a major target for HOCl [34,37], and the observations that exposure of proteins and polysaccharides to HOCl can lead to the formation of chloramines and chloramides [36,39,46]. In this study it has been demonstrated that such species are also formed on both cultured ECM, and that isolated from pig arteries, on incubation with HOCl. The loss of HOCl parallels the formation of TNB-reactive material on the ECM consistent with the latter being chloramines and/or chloramides. It is not possible to distinguish between these species with the current data. The yield of these materials is low (≤ 15%) based on the concentration of HOCl added (cf. data in Figures 2 and 7), though this may be an underestimate as a result of the instability of these materials, and the presence of these intermediates on an insoluble three-dimensional matrix which may be poorly penetrated by the large negatively charged TNB anion. The subsequent decay of these materials parallels the detection of matrix fragments as assayed by both radiolabel release (for the VSMC matrix) and measurement of uronic acid and peptide fragments (for pig artery matrix). Previous studies have shown that the decay

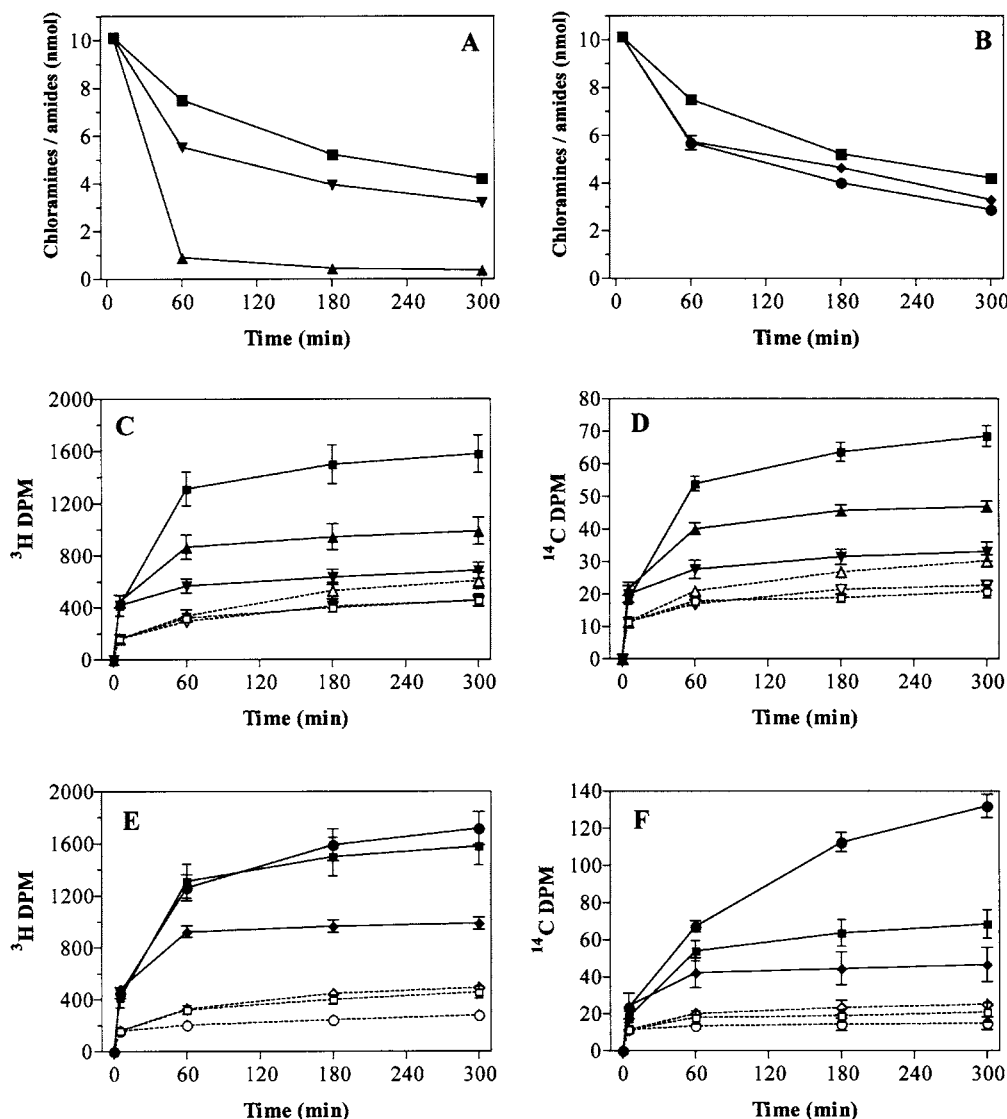


Figure 5 Effect of chloramine/chloramide scavengers and metal ions on chloramine/chloramide levels and fragmentation of smooth muscle cell ECM induced by HOCl

(A, B) Concentrations of chloramines/chloramides on ECM following incubation of ECM with 100 nmol of HOCl for 5 min and subsequent removal of excess HOCl by washing. Following such treatment the ECM was then incubated in the presence of water (■), 2.5 μmol of methionine (▲), 2.5 μmol of Trolox C (▼), 20 nmol of Cu^{2+} (●) or 20 nmol of Fe^{3+} (◆). (C, D) Radioisotope released following the above treatment protocol in either the presence (filled symbols) or absence (open symbols) of HOCl, and subsequent incubation with water (■, □), 2.5 μmol of methionine (▲, △) or 2.5 μmol of Trolox C (▼, ▽). (E, F) As for (C) and (D) except with subsequent incubation in the presence of water (■, □), 20 nmol of Cu^{2+} (●, ○) or 20 nmol of Fe^{3+} (◆, ◇). All results are means \pm S.D. ($n=6$); where no error bar is visible it is obscured by the symbol.

of chloramines/chloramides can lead to fragmentation of the proteins, due to either the formation and subsequent reactions of nitrogen-centred radicals [38,39], or hydrolysis reactions [47]. As significant fragmentation of ECM occurs long after the cessation of consumption of HOCl this degradation is consistent with the mediation of chloramines/chloramides, and this conclusion is supported by the inhibitory effects of species which are known to remove chloramines/chloramides (methionine) and/or agents (Trolox C) which scavenge radicals derived from these intermediates (Figure 5).

The time course and extent of release of the saccharide- and protein-derived materials has been shown to be markedly different with the VSMC-derived ECM. In both cases an initial rapid release of material is observed, but the proportional extent of saccharide released in this initial burst is greater than that for

the proteins. Thus approx. 75% of the total released ^3H -labelled fragments were released by 1 h, compared with 50% for the ^{14}C -labelled fragments with the VSMC ECM (Figure 4). This may indicate a greater susceptibility of the ^3H -labelled polysaccharide components to direct fragmentation by HOCl. After this initial rapid release there is a greater relative release of ^{14}C -labelled over ^3H -labelled materials over the remaining incubation period studied; this has been ascribed to fragmentation of the ECM as a result of chloramine/chloramide decay. This extended release of ECM-derived materials is exacerbated by metal ions, decreased by chloramine/chloramide scavengers, inhibited by Trolox C, and decreased in rate and extent at lower temperatures. Each of these effects is consistent with the mediation of chloramines/chloramides, and the protection afforded by Trolox C is consistent with the mediation of radical species.

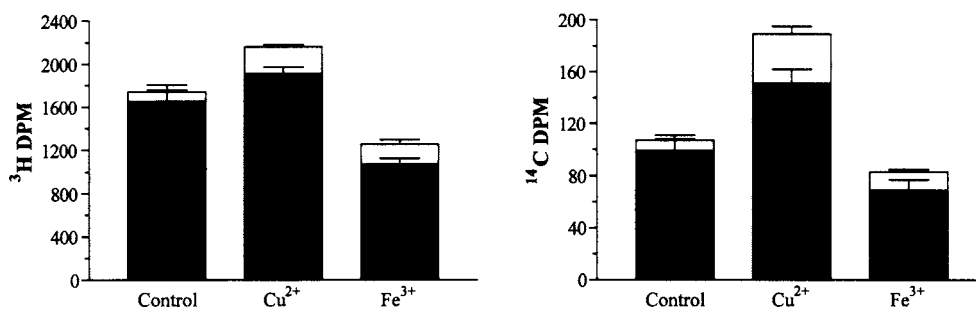


Figure 6 Release of radiolabelled fragments from smooth muscle cell ECM pre-treated with HOCl

Radiolabelled ECM was incubated with 250 nmol of HOCl in 1 ml of 50 mM phosphate buffer (pH 7.4) for 5 min at 37 °C. ECM was washed with water and incubated with water (control), or water containing 20 nmol of either Cu²⁺ or Fe³⁺, at 37 °C. Radioactivity released into solution was measured after 60, 180 and 300 min, with fresh solution being added after 60 and 180 min. After 300 min, the solution was replaced with 1 mM EDTA in water and incubated for a further 120 min at room temperature. Columns represent total radioactivity released during the first 300 min (filled bars) and subsequent release following 120 min incubation with chelator (open bars). Results are means \pm S.D. ($n = 4$). The percentage of total (420 min) radioactivity released during chelator treatment for each radionuclide is given in parentheses following the incubation condition: for ³H, water (5%), Cu²⁺ (11%) and Fe³⁺ (15%); for ¹⁴C, water (7%), Cu²⁺ (20%) and Fe³⁺ (17%).

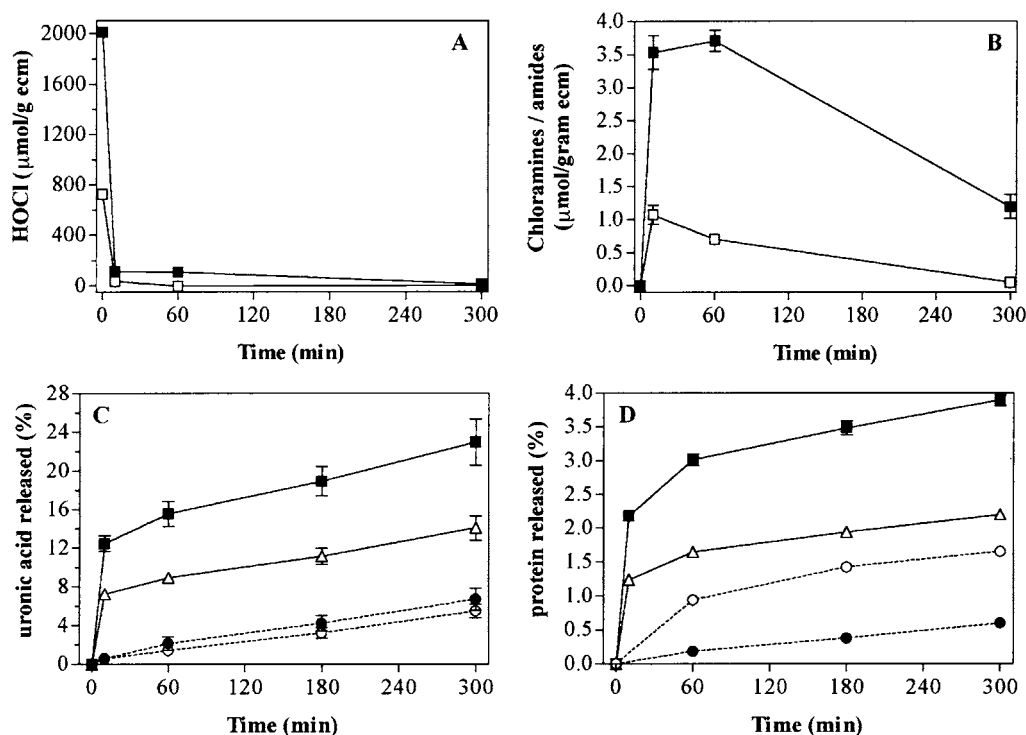


Figure 7 Consumption of HOCl, formation and decay of chloramines/chloramides, and release of uronic acid and protein from ECM isolated from pig aortae on treatment with HOCl

ECM extracted from intima of pig thoracic aortae (10 mg) was incubated with 20 (■), 10 (△) or 7.5 μmol (□) of HOCl, or no HOCl (○, ●) in phosphate buffer. (A) Consumption of HOCl from supernatant. (B) Formation and decay of chloramines/chloramides on ECM as assayed using TNB (see the Experimental section). (C) and (D) ECM was incubated in the absence (●) or presence of protease inhibitors (■, △, ○; see the Experimental section) and then HOCl in PBS (or PBS alone) for 5 min. The ECM was then washed and subsequently incubated with phosphate buffer for the remainder of experiment with uronic acid and protein measured in both the supernatant and the ECM at the indicated time points using the methods outlined in the Experimental section. Results are presented as means \pm S.E.M.; where no error bar is visible it is obscured by the symbol.

The ³H-labelled components of the ECM released by the action of HOCl are likely to be glycosaminoglycan-derived materials, arising from damage to proteoglycans. This is in accord with previous studies which have shown that proteoglycans associated with endothelial cells are susceptible to cleavage by activated neutrophils [48]. The majority of glycosaminoglycans incorporated in proteoglycans are attached via Ser and Thr residues which are particularly abundant in the protein core of proteoglycans, thereby allowing multiple glycosaminoglycan attachments [49]. Previous studies have shown that proteoglycans

from human atherosclerotic plaques have fewer polysaccharide chains than adjacent normal human aorta [5], in accord with our observations on the behaviour of both the ECM synthesized by VSMCs *in vitro*, and ECM extracted from porcine thoracic aorta, when treated with HOCl. Although glycosaminoglycans lack many of the HOCl-reactive functions that are found in proteins, the large number of N-acetyl functions present on these polymers may make these a major target for HOCl, with subsequent fragmentation via chloramide formation and decay ([36] and M. Rees, C. L. Hawkins and M. J. Davies, unpublished work). The

nature of the radical-mediated reactions which bring about the fragmentation of glycosaminoglycans has recently been examined in detail, and evidence presented for a key role of hydrogen-atom abstraction reactions initiated by *N*-acetyl chloramide-derived amidyl radicals (M. Rees, C. L. Hawkins and M. J. Davies, unpublished work).

A previous study by Davies and colleagues [50] has reported that *N*-chloramines do not cause direct fragmentation of collagen, but instead greatly increase the susceptibility of this material to degradation by collagenase and elastase. In the studies reported here significant proteolytic degradation of ECM synthesized by VSMCs *in vitro* was not observed in the absence of HOCl (Figure 3). Such a result is not unexpected due to the treatment of the ECM with NH₄OH and subsequent multiple washings (see the Experimental section), which would be expected to remove such enzymes. It is therefore unlikely that enzymic proteolysis is a significant factor in these studies, and that the fragmentation observed in the presence of HOCl and chloramines/chloramides arises via these species. In the corresponding experiments with ECM extracted from porcine aorta, the ECM was pre-treated with irreversible inhibitors which, in the absence of HOCl, were shown to significantly inhibit protein release (Figure 7). However even in the absence of such inhibitor treatment, only limited direct proteolysis was observed (Figure 7), with increased protein degradation observed in the presence of chloramines and chloramides. As ECM proteins such as collagen and elastin are rich in proline, the release of incorporated ¹⁴C from the ECM observed on treatment with HOCl can be, at least partly, attributed to the fragmentation of these species. In the absence of inhibitors of matrix-degrading enzymes, it is impossible to delineate, with the current data, the role of direct HOCl/chloramine/chloramide-mediated fragmentation from the potential role of HOCl in activating latent matrix-degrading enzymes; such enzymes are known to be both activated and inactivated by HOCl [51]. Matrix degradation *in vivo* by HOCl (and species derived from this) is therefore likely to occur via multiple, interacting, mechanisms.

We observed marked differences in the ability of methionine and Trolox C to quench chloramines/chloramides on HOCl-treated ECM. Similar results have been observed for HOCl-treated plasma proteins [38] and may relate to the higher concentrations employed, and faster rate of reaction, of methionine, when compared with Trolox C, with HOCl [37,52]. However, in marked contrast to the results of the study with plasma proteins, Trolox C had a greater inhibitory effect on fragmentation than methionine, for both ³H- and ¹⁴C-radiolabelled components of the ECM. Whereas some of this difference may arise from the slight stimulation of fragmentation induced by methionine on untreated ECM (cf. Figures 5C and 5D), this may also arise as a result of other effects. Thus Trolox C is known to be a very efficient radical scavenger for a range of radicals [53], whereas methionine only reacts rapidly with some oxidants (e.g. hydroxyl radicals [54]) and not others. The more marked effect of Trolox C may therefore arise via the ability of this compound, but not methionine, to remove radicals formed as a result of chloramine/chloramide decay. This hypothesis is supported by a previous study which has reported that Trolox C is an efficient scavenger of amino acid- and peptide-derived nitrogen-centred radicals derived from chloramines/chloramides, with rate constants in the range 10⁷–10⁸ M⁻¹ · s⁻¹ [55]. Furthermore evidence has also been obtained for the formation of radicals on decomposition of ECM-derived chloramines/chloramides, generated by HOCl, by use of EPR spin trapping (B. Brown and M. J. Davies, unpublished work).

Metal ions have previously been shown to accelerate chloramine and chloramide degradation on amino acids, proteins and glycosaminoglycans [36,47,56] and a similar effect has

been observed in the current study with intact HOCl-treated ECM. However, marked differences were observed in the rate and extent of release of radiolabelled fragments from the ECM between the two metal ions examined. This effect may in part be explained by the precipitation or binding of ECM fragments by the metal ions. Our observation that EDTA treatment, following HOCl and metal ion incubation, released proportionally greater amounts of radioactivity from the ECM than the no-metal-ion-treated controls, suggests that significant binding of fragments was occurring. Previous studies have reported that iron accumulates in human atherosclerotic plaques ([57,58] and R. Lindner, N. Stadler and M. J. Davies, unpublished work), and our study demonstrates that Fe³⁺ can bind such fragments to the ECM.

The formation of fragments from both protein and polysaccharide components of the ECM may have profound effects on surrounding cells [59], as has been observed with other low-molecular-mass materials derived from glycosaminoglycans [60–63]. The effects of such modified matrix materials on cellular behaviour are currently poorly understood, though it is known that changes in (non-modified) matrix materials can have profound effects on cell adhesion, proliferation, growth and cell phenotype [44], as can oxidation of normal matrix components [64]. The fragmentation and other modifications of ECM reported in the current study may therefore be of considerable significance in diseases such as atherosclerosis [65] where marked changes in cellular behaviour are observed under circumstances where MPO-derived oxidants are known to be generated [15,20].

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