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Vitamin C induced decomposition of lipid hydroperoxides: direct evidence of genotoxin–DNA binding detected by QCRS

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We report direct evidence for human DNA-binding to precursors of mutagenic lesion-inducing compounds, produced form the vitamin C induced decomposition of lipid hydroperoxides, using a quartz crystal resonant sensor assay.

DNA damage has been shown to occur from reactive oxygen species (ROS),[1] or from ROS derived lipid hydroperoxides breaking down to form endogenous genotoxins.[2,3] Ascorbic acid (vitamin C) is a widely ingested dietary supplement that has long been advocated as an antioxidant due to its ability to scavenge reactive oxygen species,[4] although it has also been reported to exhibit pro-oxidant behaviour in the presence of transition metal ions. It has very recently been shown that decomposition of lipid hydroperoxides, such as (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadecan-9,11-dienoic acid ((13*S*)-HPODE), occurs in the presence of excessive quantities of ascorbic acid[5] exhibiting time-dependent production of a range of compounds including 4-oxonon-2-enal, a well established genotoxin.[6] It was proposed that the efficiency of ascorbic acid in inducing the decomposition could lead to significant DNA damage *in vivo*,[5] however this hypothesis remained untested.

A human DNA binding and damage assay has recently been developed in our laboratory based upon quartz crystal resonant frequency sensor (QCRS) technology.[7] QCRS sensors are rapidly emerging technologies incorporating quartz crystals as sensing transducers, and have shown great promise for label-free monitoring of solution phase surface interactions.[8] Acoustic sensors of this type have been employed for a diverse range of applications including antibody agglutination,[9] RNA interactions[10] and protein-ligand binding.[11] It was originally thought that sensors operating in this mode of action were susceptible to frequency changes caused solely by mass deposition on the sensor surface. More recently it has been shown that factors other than mass, such as density and viscosity or other viscoelastic effects, may also influence the frequency changes observed.[12] QCRS technologies have been successfully used to measure ligand DNA binding by ourselves and others.[7,13] Herein we use it to demonstrate direct DNA binding from the mixture of compounds generated by the vitamin C induced decomposition of the lipid hydroperoxide (13*S*)-HPODE.

Experiments were performed upon human DNA bound to the surface of a quartz crystal resonant sensor using a bifunctional linker.† Decomposition reactions were performed in accordance with the timescales and concentration ranges given in the existing literature.[5] Products of the decomposition reaction mixtures were tested for their DNA binding or damaging characteristics and compared with known DNA binding compounds, intercalators and damage inducers.

Molecular ion determination by MALDI-TOF mass spectrometry of the 100 μM incubation detected 4-hydroperoxynon-2-enal (4-HPNE) following a 30 min incubation.‡ With increased incubation time (120 min) a reduction in this product and the concomitant appearance of new species was observed. Mass spectral analysis confirmed the presence of 4-oxonon-2-enal (4-ONE), 4-hydroxynon-2-enal (4-HNE), and 4,5-*trans*-(2*E*)-4,5-epoxy-dec-2-enal (*t*-4,5-EDE) in agreement with Lee *et al*.[5] Increased ascorbic acid concentration (2 mM) did not produce 4-HPNE following a 30 min incubation, however, 4-ONE, 4-HNE and *t*-4,5-EDE were all detected. Increasing the incubation time to 120 min at this concentration led to the production of 4,5-EDE although the isomer, *cis* or *trans*, could not be confirmed.

Control experiments using the QCRS instrument led to frequency–time profiles of the type shown in Fig. 1. For both 100 μM and 2 mM ascorbic acid, initial frequency decreases were observed upon introduction of the material to the flow cell followed by subsequent frequency increases as material was washed from the chamber. The magnitude of the frequency decrease was almost equal at both concentrations suggesting that a slight rearrangement of the surface bound DNA took place in the presence of ascorbic acid.

An overall small residual frequency decrease of 10 ± 2 Hz was observed in both cases which suggests that the localised presence of ascorbate led to a reconfiguration of the bound DNA. The similar residual shift observed in both cases implies that a very small quantity of ascorbic acid was required to register an effect on the sensor.

Analysis of the incubation mixtures containing (13*S*)-HPODE led to distinct differences in the frequency–time profiles. Introduction of 30 min incubations at both 100 μM and 2 mM ascorbic acid led to very distinct and rapid concentration dependent frequency increases within the first 400 s; the higher concentration is illustrated in Fig. 1. These increases were followed by much slower decreases leading to residual frequency increases of between 5 Hz (100 μM) and 20 Hz (2 mM). The rapid frequency increase suggests a significant alteration in the structure of the bound DNA leading to

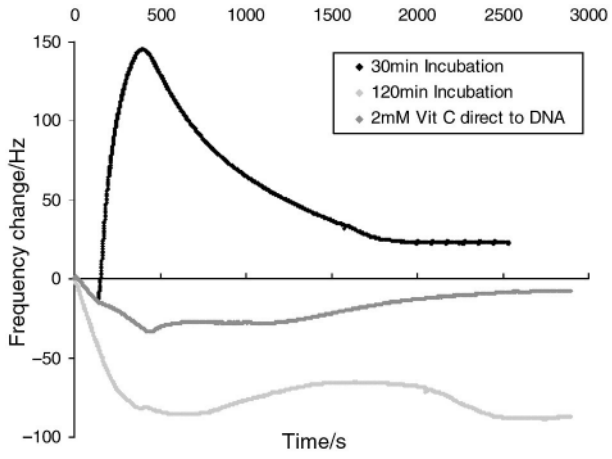

Fig. 1 Frequency–time profile for the response of QCRS-bound human DNA to ascorbic acid (vitamin C) and the decomposition mixtures of ascorbic acid (2 mM) incubated with (13*S*)-HPODE for periods of 30 and 120 min (flow rate 5 μl min^{-1}, pH 7.0, 37 °C).

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viscoelastic changes at the surface. The residual shifts in both cases suggests that either some of this structural change remains after removal of the incubation material or that 4-HPNE is binding directly to the DNA causing a transient change in structure followed by removal of some material from the surface. We have shown that similar experiments, performed in the presence of a DNA cleaving agent such as bleomycin[14] show an increase in resonant frequency indicating removal of material from the sensor surface. Similar frequency increases, although generally rare, have been observed upon the binding of ligands to RNA.[10]

At a higher ascorbic acid concentration (2 mM) and longer incubation time (120 min) a very different frequency–time profile was observed when degradation mixtures were introduced to DNA coated crystals. Fig. 1 shows a very distinct, large frequency decrease of ~85 Hz in the initial 400 s followed by a transient frequency increase before arriving at a residual, stable 90 Hz decrease. This is consistent with strong covalent binding of small molecules, such as cisplatin,[7] to DNA. In this experiment it confirms the formation of a DNA adduct with one or more components of the degradation mixture.

When a short incubation period is employed with the 2 mM concentration, the residual increase in frequency agrees with previous experiments where DNA has been shown to be removed. This is less obvious from the frequency–time profile for the mixture generated from the longer incubation periods at high concentration where the overall large frequency decrease due to binding undoubtedly masks any small frequency increase due to DNA removal. A 30 min incubation period has been shown to generate 4-HPNE as the major product whereas a wider range of products is formed at 120 min. More than one species may be responsible for the binding and/or damaging mechanism, masking the frequency increase in the case of the higher concentration due to the overall magnitude of the interaction.

In this study we have independently confirmed the presence of potentially genotoxic species in the incubation liquor produced when concentrations of ascorbic acid equivalent to extreme daily doses are brought into contact with the lipid hydroperoxide (13*S*)-HPODE. We have also shown conclusively for the first time that when mixtures of the breakdown products from these incubations are brought into contact with human DNA, following short incubation times, an effect equivalent to a refolding or reorganization of the DNA occurs. Longer incubation periods yield (2*E*)-4,5-epoxydec-2-enal, amongst other products, which unequivocally binds to DNA in a manner shown previously to be similar to that of agents such as cisplatin. A further DNA reorganization step is also suggested which to date has not been observed with the well-defined compounds in our previous assay.[7] Further work is required with deconvoluted incubation mixtures in order to ascertain the order and mechanism of binding for each component of the mixture.

It would appear that the long-term usage of extremely high doses of vitamin C leads to the production of species which bind to human DNA, however, our results are based on interactions with tethered DNA and may not represent the effects of vitamin C *in vivo*. The potential for the ascorbic acid–(13*S*)-HPODE system to access DNA in whole cells remains to be shown.

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Notes and references

† Crystals for the QCRS DNA-binding assay were prepared as previously described.[7] Samples of ascorbic acid, incubated for either 30 or 120 min with (13*S*)-HPODE at 37 °C, were introduced to the flow cell *via* an all PEEK™ Rheodyne MMB six way injection valve in line with the flow cell. Modified electrodes were exposed to solutions of the (13*S*)-HPODE degradation products and resultant frequency changes monitored over time. Control experiments to investigate the effect of ascorbic acid only upon DNA were carried out alongside the incubations at equivalent concentrations. Care was taken in all experiments to exactly match the running buffer constituents to those of the injected samples in order to remove the possibility of frequency change due to viscosity or density differences between the phases.

ACS grade ascorbic acid, (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoic acid (13*S*)-HPODE), dihydrogen potassium phosphate, disodium hydrogen phosphate, sodium chloride and HPLC grade ethanol were purchased from Sigma-Aldrich (Poole, Dorset, UK). Buffers for all solutions were prepared using 18.2 MO metal free, reverse osmosis HPLC grade water (Elga Purite, Elga, UK). Incubations of ascorbic acid at concentrations equivalent to dosages of 60 mg per day (100 μM) and 2000 mg per day (2 mM) were made with (13*S*)-HPODE for periods of 30 and 120 min at 37 °C, as described in the original study.[5]

‡ Characterisation of the incubation solutions was performed using MALDI-TOF mass spectrometry in a cobalt matrix (Kratos PCKompact, Kratos, UK). (13*S*)-HPODE degradation products were detected from *m*/*z* values with 2 dp accuracy [100 μM ascorbic acid, 30 min: 4-hydroperoxynon-2-enal (4-HPNE, 156); 100 μM ascorbic acid; 120 min: 4-oxonon-2-enal (4-ONE, 155), 4-hydroxynon-2-enal (4-HNE, 157), 4,5-*trans*-(2*E*)-4,5-epoxydec-2-enal (*t*-4,5-EDE, 169); 2 mM ascorbic acid, 30 min: 4-ONE (155), 4-HNE (157), *t*-4,5-EDE (169); 2 mM ascorbic acid, 120 min: 4,5-EDE (169)].

Eluent from the QCRS flow cell, following addition of the ascorbic acid–(13S)-HPODE decomposition mixtures was collected and examined by UV spectroscopy (Shimadzu UV-1601) at 260 nm for the presence of human DNA.

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