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Nicotine-enhanced oxidation of low-density lipoprotein and its components by myeloperoxidase/H_2O_2/Cl^- system

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ABSTRACT

In this study, the effect of nicotine on the LDL oxidation by the MPO/H_2O_2/Cl^- system and the effect of HOCl on LDL and some of its components, such as methyl linoleate, vitamin E and the amino acid tryptophan were explored. Nicotine, in micromolar concentrations, enhanced the tryptophan oxidation, either present in LDL or free, in solution. Nicotine also decreased the formation of conjugated dienes and oxygen consumption in a methyl linoleate / HOCl system, and there was evidence to suggest an increase in chlorohydrin formation. Acceleration of the vitamin E oxidation by HOCl was also observed in the presence of nicotine. These data show that the interaction of nicotine and HOCl can promote significant biochemical modifications in LDL particle and some of its components involved in the pathogenesis of cardiovascular and other diseases.

Key words: nicotine, myeloperoxidase, hypochlorous acid, low-density lipoprotein, methyl linoleate, vitamin E, tryptophan.

INTRODUCTION

Hypochlorous acid (HOCl) is a major oxidant produced by neutrophils, in a reaction catalyzed by myeloperoxidase (MPO; EC 1.11.1.7), a heme enzyme present in high concentrations in the granules of leukocytes (Winterbourn and Kettle 2013). MPO uses hydrogen peroxide (H_2O_2), produced during the oxidative burst, to oxidize Cl^- to HOCl, by the following reactions (Vlasova et al. 2012):

$$\text{native MPO} + H_2O_2 \rightarrow \text{MPO I} + H_2O$$

$$\text{MPO I} + Cl^- \rightarrow \text{native MPO} + \text{HOCl}$$

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HOCl is a potent oxidizing agent that plays a cytotoxic role against invading bacteria, viruses, and tumor cells and can also cause oxidation of proteins, nucleic acids and lipoproteins *in vivo* (Sassa et al. 2013). Recent studies have shown the possibility of MPO as an independent risk factor for the development of atherosclerosis (Salonen et al. 2012, Alipour et al. 2013). Nicotine is an alkaloid composed of a pyridine and a pyrrolidine ring and is one of the components of tobacco (Clayton et al. 2013). It is known that nicotine induces oxidative stress both *in vitro* and *in vivo* (Newman et al. 2002). It has been proposed that tertiary amines, such as nicotine, trimethylamine

and quinine, react with HOCl to form reactive Cl^+ adducts (R_3N^+ - Cl, reactive quaternary chlorammonium ions) that readily release Cl^+, enhancing the formation of chlorinated/oxidized products (Prutz 1998, Suzuki and Ohshima 2002). It is also suggested that the interaction between significant concentrations of nicotine (in smokers) and HOCl may promote DNA (Masuda et al. 2001) and tissue damage (CV-1 mammalian kidney cells; Salama et al. 2014).

Cardiovascular disease is currently the leading cause of morbidity and mortality worldwide and its incidence is likely to increase. Many studies have indicated that MPO is an inflammatory marker in coronary artery disease (Zhang et al. 2001); in particular, due to its ability to generate reactive oxygen species, which promote oxidative damage to lipoproteins and leading to progression of atherosclerosis (Haraguchi et al. 2014).

In light of such reports on the properties of R_3N^+ - Cl and the possibility of its *in vivo* generation with nicotine, and considering their relation to oxidative stress, we explored the effect of nicotine on the LDL oxidation by the MPO/H_2O_2/Cl^- system and the effect of HOCl on LDL lipid components, methyl linoleate (ML), vitamin E and tryptophan.

MATERIALS AND METHODS

Reagents

MPO of a purity index (A_{430} / A_{280}) of at least 0.85 was purchased from Planta Naturstoffe Vertriebs GmbH. H_2O_2 (stocked as a 30% solution) and NaOCl, both from Merck, were diluted shortly before use, the concentration of H_2O_2 being determined by measuring absorbance at 230 nm (ϵ = 80 M^{-1} cm^{-1}; Brestel 1985) and that of OCl^- at 292 nm (ϵ = 350 M^{-1} cm^{-1}; Zgliczynski et al. 1971). Nicotine tartrate was purchased from Sigma (St. Louis, MO, USA). All other reagents were analytical grade, obtained from Sigma or Aldrich.

Isolation of LDL

Venous blood from "healthy" volunteers (non-fasting, age range 23–50 years, non-smokers) was collected into tubes containing ethylenediaminetetraacetic acid (1.0 mg/mL of blood). Plasma was separated by centrifugation at 1000 × g for 10 min, at 4 °C, and 5.0 μM phenylmethylsulfonyl fluoride, 10.0 μM benzamidine, 10.0 μg/mL aprotinine and 100.0 μM butylated hydroxytoluene were added to prevent protease activity and oxidative reactions. The study was approved by the research ethics committee (FCF/UNESP: protocol CEP/FCF/Car. N 02/2005 and document 022/2005). Firstly, very-low-density lipoprotein (VLDL) was separated: the plasma was placed in ultracentrifuge tubes with saline solution (about 60% of the total volume) and rotated at 55,000 g, for 7h at 4 °C. At the end of this period, the VLDL floated and was drawn off with great care. The remaining plasma was transferred to a test tube, where its density was adjusted to 1.063 g/L with solid KBr. It was then centrifuged again at 55.000 g for 7h, at 4 °C, bringing to the surface the LDL, which was delicately removed and dialyzed in phosphate-buffered saline in 20 mM sodium phosphate, pH 7.4 and 0.14 M NaCl, for 4h at 4 °C, the buffer being renewed every hour. The LDL was stored at 4 °C in the dark, for a maximum of 2 weeks, until used (Bagheri et al. 2013). Protein content was determined by the Lowry method (Lowry et al. 1951).

LDL Oxidation by MPO/H_2O_2/Cl^- System

LDL (80 μg protein/ml) was oxidized by the system MPO (1 nM) / H_2O_2 (0.5 mM) in 50 mM sodium phosphate buffer (pH 7.4) with 0.14 M Cl^-, at 25 °C. This reaction was monitored by the fall in fluorescence (excitation at 282 nm and emission at 331 nm). The LDL α-tocopherol fluorescence (excitation at 290 nm and emission at 323 nm) does not interfere with the tryptophan fluorescence even at high concentrations (Jerlich et al. 1998).

Tryptophan Oxidation by MPO/H_2O_2/Cl^- System

Tryptophan (0.1 mM) was oxidized by the system MPO (1.0 nM) / H_2O_2 (0.25 mM) in 50 mM sodium acetate buffer (pH 5.5) with 0.14 M Cl^-, at 30 °C. The reaction was monitored by following the decay in fluorescence of the tryptophan (excitation at 290 nm and emission at 360 nm; Jerlich et al. 1998).

Methyl Linoleate (ML) Oxidation by HOCl

A 50 mM solution of sodium dodecyl sulphate (SDS) was made in 50 mM sodium phosphate buffer (pH 7.4). 20 µL of ML (density 0.9 g/mL) was added and the mixture shaken until it had all dissolved (to a transparent solution of final concentration 1.2 mM). These solutions were prepared with and without nicotine, and the ML was oxidized at 37 °C by adding HOCl. The reaction was monitored by following (i) the formation of conjugated dienes at 234 nm and (ii) the consumption of oxygen; both assays were conducted in a thermostatic cuvette (Noguchi et al. 2002).

Vitamin E Oxidation by HOCl

The reactions of HOCl with vitamin E, in the absence and presence of nicotine, were carried out in 50 mM sodium phosphate buffer (pH 7.4) with SDS 1%, at 37 °C. The absorbance at 255 nm and spectral variation were monitored, at intervals of 1 second, using a diode-array spectrophotometer. The reactions were started by adding HOCl (Pattison et al. 2003).

HOCl Determination

A solution containing 14 mM 3,3',5,5'-tetra-methylbenzidine (TMB) dissolved in 50% dimethylformamide, 100 mM potassium iodide and 400 mM acetic acid was used to measure HOCl. Under these conditions HOCl oxidizes TMB to a blue product with an absorbance maximum at 655 nm. A standard curve was generated by adding pure HOCl (Dypbukt et al. 2005).

Tartrate Blank

All assays performed with nicotine tartrate were repeated with sodium potassium tartrate and with buffer alone. No difference was observed between these tartrate and buffer solutions.

Statistics

Statistical analysis of the data was performed via one-way analysis of variance (ANOVA). The results were considered statistically significant if $p < 0.05$.

RESULTS

Effect of Nicotine on the Oxidation of Free and LDL Tryptophan by MPO/H_2O_2/Cl^- System

Using this model system the oxidation of free tryptophan was observed as a gradual fall in the fluorescence intensity. In Figure 1A, it can be seen that the MPO/H_2O_2/Cl^- system promoted tryptophan oxidation and that increased concentrations of nicotine led to a marked progressive rise in the fluorescence decay, in which represents an increase in the oxidation of tryptophan (evaluated by rate constant for the oxidation of tryptophan, measured by tangent of fluorescence decay curve). A similar effect was observed in the fluorescence of LDL particles exposed to the MPO/H_2O_2/Cl^- system, which fluorescence decay was also enhanced by nicotine in the reaction medium (Fig. 1B).

In both the above experiments, the effect of the enzymatic reaction on the LDL or free tryptophan, was only seen with the complete system, MPO/H_2O_2/Cl^-, indicating that the HOCl is responsible for the loss of fluorescence, as previously postulated by others (Jerlich et al. 2000a).

Effect of Nicotine on the Oxidation of the ML by HOCl

Figure 2A demonstrates that HOCl provoked an effective rise in absorbance at 234 nm, due to ML oxidation, and that in the presence of nicotine (0.1 and 0.5 µM), there was a fall in the rate of formation of conjugated dienes. At the highest nicotine concentration used (1.0 µM), there was actually a clear fall in absorbance at 234 nm, providing strong evidence of the chlorination of the double bounds in the ML molecule, which would cause

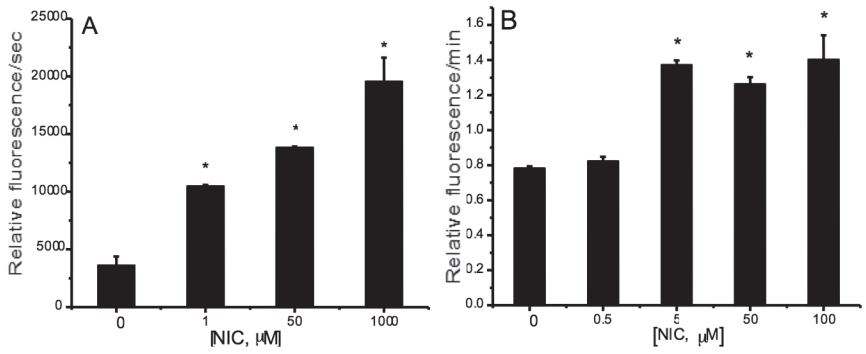

Figure 1 - A) Effect of nicotine on the rate of free tryptophan oxidation by the MPO/H_2O_2/Cl^- system. Tryptophan (0.1 mM) was incubated with MPO (1.0 nM) in 50 mM sodium acetate buffer (pH 5.5), containing 0.14 M NaCl, at 30 °C. The reaction initiated adding 0.25 mM H_2O_2, in the absence or presence of various concentrations of nicotine (Mean ± SD; n=5) . (B) Rates of tryptophan oxidation in the LDL particle. LDL (80 μg protein/mL) was incubated with MPO (1.0 nM) in absence or presence of various concentrations of nicotine, in 50 mM sodium phosphate buffer (pH 7.4) containing 0.14 M NaCl, at 25 °C. The reaction initiated adding 0.5 mM H_2O_2 (Mean ± SD; n=6). * Significantly different from control ($p < 0.05$).

such a fall in absorbance. This fall cannot be due to a suppression of HOCl by nicotine, since, up to 1.5 μM, nicotine exhibited virtually no scavenging effect on HOCl (Fig. 2B).

In parallel to the formation of conjugated dienes, the reaction between HOCl and polyunsaturated fatty acids (PUFA) generates chlorohydrin. This reaction is well characterized for both PUFA and cholesterol (Winterbourn et al. 1992). The mechanism by which chlorohydrin is formed involves two electrophilic addition reactions: the Cl^+ ion is added at the double bond and then OH^- is added at the other carbon atom of the same bond. If the loss of double bonds outstrips the formation of new conjugated dienes, a reduced rate of change will be observed in the absorbance at 234 nm. Arnhold et al. (1995), showed that there was a direct relation between the amount of HOCl consumed and the loss of double bounds of linoleic acid, which resulted in the formation of chlorohydrins. The production of these compounds may occur in certain biological events and, as they are more polar than their lipid precursors, their appearance in cell membranes might cause changes in the membrane structure and consequently in its functionality (Winterbourn et al. 1992). In the case of cholesterol chlorohydrin, the possibility has been discussed that it may cause harmful effects in artery walls (Heinecke et al. 1994); futhermore, it has been shown to promote the lysis of red blood cells (Vissers et al. 1994).

Our results show that there may be an increased formation of chlorohydrin in the interaction of the HOCl/ML system with nicotine. Thus, an experiment was done to determine the variation in the concentration of HOCl (in presence of ML) in the reaction medium, *via* oxidation of TMB. Nicotine, in micromolar concentrations, accentuated the depletion of the HOCl, compared to the control: 0.25 - 1.5 μM nicotine led to 80-100% decreased oxidation of TMB, while the assay without nicotine showed a fall of only 38% (Fig. 2B). In order to visualize this effect directly, the ML/HOCl reaction was followed by measuring absorbance in the region 200-300 nm; an effective fall can be seen in absorbance at 234 nm (conjugated dienes) and at 292 nm (HOCl absorption peak) in the presence of nicotine (Fig. 3A) relative to the assay without nicotine, with a fall at 292 nm and rise at 234 nm (Fig. 3B).

The oxidation of PUFA leads to the consumption of the equivalent amount of O_2 from the reaction medium, as hydroperoxides are formed. The consumption of oxygen has been used to monitor the oxidation of ML in micelles (Rossetto et al. 2002).

The oxygen depletion profile of the HOCl/ML in SDS micelles, in the absence and presence of nicotine, is displayed in Figure 3C. The HOCl promotes the consumption of O_2 from the reaction mixture with ML and it can be seen that nicotine at 1 and 2 μM significantly inhibits the O_2 consumption. Nicotine, at these low concentrations, has an insignificant scavenging effect on HOCl, and therefore it seems likely that another mechanism, different from conjugated-diene formation, is responsible for this effect.

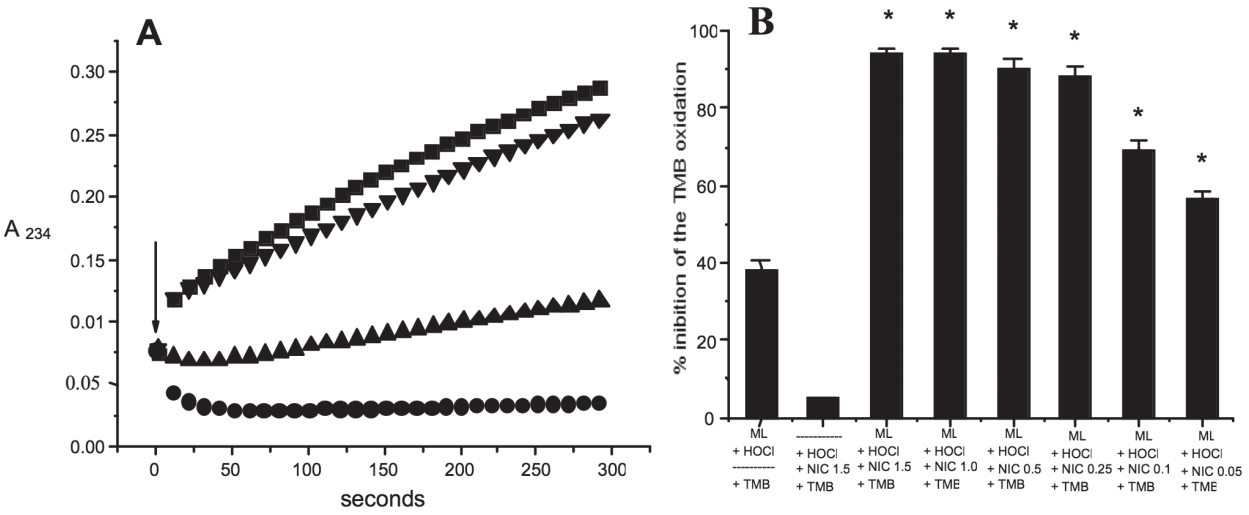

Figure 2 - A) Effect of nicotine on the ML oxidation by HOCl. 1.3 mM ML, 1.0 mM HOCl and 0 (■), 0.1 (●), 0.5 (▲) or 1.0 (▼) μM nicotine in 50 mM sodium phosphate buffer, (pH 7.4), with 50 mM SDS at 37 °C. The arrow shows the initial absorbance, before addition of HOCl. B) Effect of nicotine on the TMB oxidation by HOCl in the presence of ML. Nicotine (NIC, μM) and ML (1.3 mM) were incubated for 1 min and 0.5 mM HOCl was added, in a medium containing 50 mM SDS in phosphate buffer (pH 7.4). After 5 min, the remaining HOCl was estimated by adding 2.8 mM TMB and monitoring absorbance at 652 nm (Mean ± SD; n=5). * Significantly different from control (the sum of % inhibition of TMB oxidation by the systems ML/HOCl and nicotine/HOCl), with $p<0.05$.

Effect of Nicotine on the Vitamin E/HOCl System in SDS Micelles

In Figure 4A, it can be seen that in the presence of nicotine there is a rise in A_{240nm}, relative to the vitamin E/HOCl reaction without nicotine. Concentrations of 1 and 5 μM of nicotine caused a strong and fast rise in A_{240nm}. Figure 4B and C also show the absorption spectrum from 220 to 350 nm, and how it develops during the reaction, in the absence or presence of nicotine. The accentuated increase in absorption between 220 and 280 nm may correspond mainly to the formation of a vitamin E dichloroquinone (Ho et al. 2000).

DISCUSSION

The great majority of experiments on LDL oxidation are conduced "in vitro". In this case, the oxidation reaction can be followed by observing changes in the physicochemical and biological properties of the LDL (Jerlich et al. 1998, 2000a).

It is well established that the apoproteins (apo B-100 being the main one in LDL) contain the aromatic residues of tyrosine and tryptophan which contribute visibly to the intrinsic fluorescence of the LDL particle. During LDL oxidation, there is a significant fall in the fluorescence of tryptophan and this is used in the investigation of *in vitro* model systems. As a

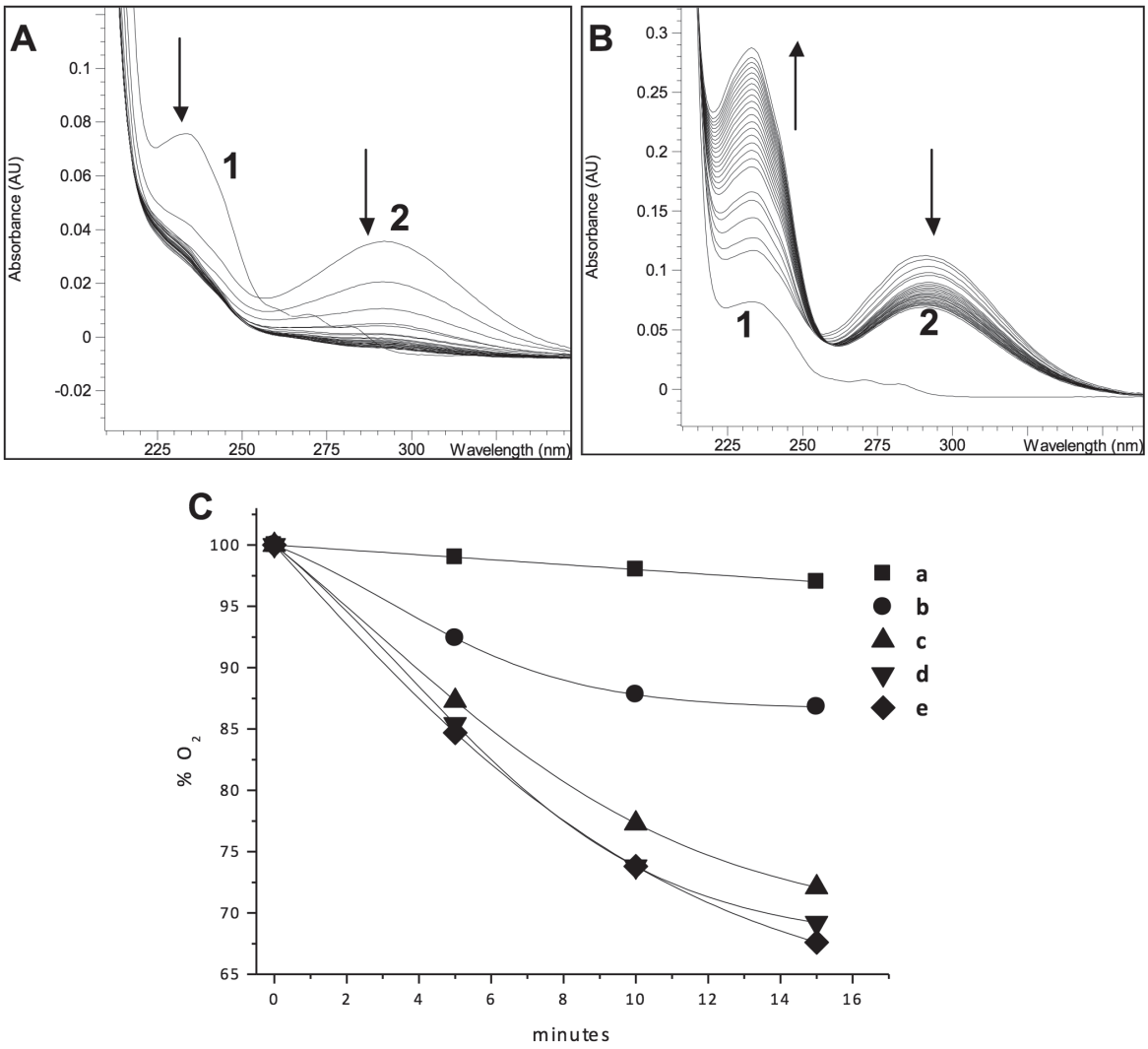

Figure 3 - Effect of nicotine on ML oxidation: (1) ML (1.3 mM) and (2) immediately after addition of HOCl (1 mM), as they develop at 10-second intervals, in the directions indicated by the arrows. A, nicotine absence and B, with 1.0 µM nicotine. The reaction medium was 50 mM sodium phosphate buffer (pH 7.4), with 50 mM SDS at 37 °C. C) Oxygen depletion during oxidation of ML by HOCl. 10 mM ML was prepared in 50 mM sodium phosphate buffer (pH 7.4) with 50 mM SDS. Reaction initiated adding 1.0 mM HOCl, at 37 °C, in the absence or presence of nicotine. a) ML and nicotine absent; b) ML, HOCl and 2.0 µM nicotine; c) ML, HOCl and 1.0 µM nicotine; d) ML, HOCl and 0.2 µM nicotine; e) ML and HOCl.

consequence of the oxidation of tryptophan residues, the structure of LDL suffers modification, affecting its biological properties, such as its binding to receptors on cells (Jerlich et al. 2000a).

Oxidative reactions convert tryptophan to kynurenine, *N*-formylkynurenine, oxindolylalanine and hydroxytryptophan, altering both the structure and function of the protein (Thomas and Stocker 1999). Tryptophan is oxidized by HOCl, and its fluorescence significantly decreases when lipoproteins are exposed to the MPO/H_2O_2/Cl^- system. The indole ring of tryptophan initially reacts with HOCl to yield a chlorinated species, which is likely to be 3-chloroindolenine or perhaps the *N*-chloroindole species (Fu et al. 2006).

The presence of PUFA makes the membrane susceptible to oxidation processes known as lipid peroxidation (Gasparovic et al. 2013).

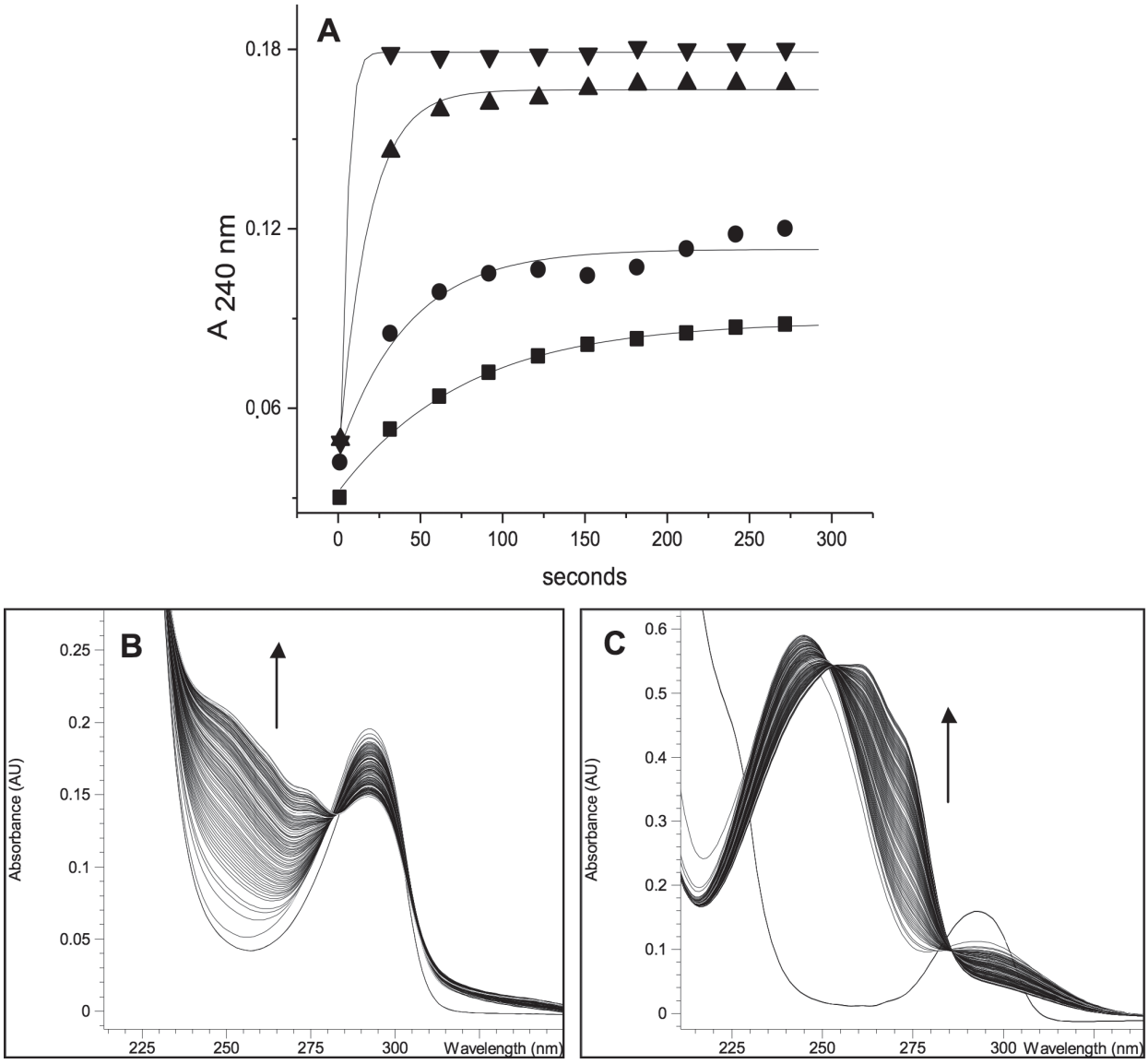

Figure 4 - Effect of nicotine on the oxidation of vitamin E (5 mM) by HOCl (0.05 mM) in 50 mM sodium phosphate buffer (pH 7.4) with 50 mM SDS at 37 °C. (A) Reaction time-course: without nicotine (■); with 0.1 (●), 1.0(▲) or 5.0 (▼) μM nicotine. Spectral variation during reaction at 1 second intervals (arrows show increasing time) in the absence (B) or presence (C) of 1 μM nicotine, the number 1 shown the vitamin E spectrum in the reaction mixture before the HOCl.

The radicals produced, undergo rearrangements to form conjugated dienes. These molecules react with O_2, generating peroxide radicals that, in turn, can abstract another H atom to form hydroperoxides, thus propagating the chain of reactions. This process leads to the formation of secondary products such as alcohols, ketones and aldehydes. The formation of conjugated dienes has been widely studied by UV absorption spectroscopy, to be used as a marker for lipid peroxidation (Noguchi et al. 2002, Rekdal and Melo 1995).

LDL contains PUFA in its structure, and these molecules may be a target for oxidative damage during atherosclerosis; thus, the effects of HOCl on these moieties have been investigated in a number of studies (Spiteller 2005).

One such mechanism might be chlorohydrin formation in the ML/HOCl/ nicotine system. As can be seen, nicotine leads to a fall in absorbance at 234 nm, which must be due to the loss of one or more double bonds. Chlorohydrin formation in this system would result in a smaller proportion of conjugated dienes, which in turn would diminish the rate of consumption of dissolved oxygen. It is worth remembering that this effect could occur anywhere in the body where nicotine is found, together with PUFA and HOCl generation. Chlorinated lipoproteins have been located in human atherosclerotic lesions (Malle et al. 2006), so a relevant factor might be the effect of nicotine on the oxidation of LDL, which is rich in PUFA, by HOCl or the MPO/H_2O_2/Cl^- system, making that oxidation potentially more atherogenic. It is important to note that chlorohydrins are the main products of the reaction between HOCl and lipids in relation to the formation of hydroperoxides (Jerlich et al. 2000b).

Vitamin E (tocopherol) is an important antioxidant in the diet, the main lipophilic antioxidant in plasma, membranes and tissues and also an important inhibitor of lipid peroxidation that blocks the propagation of this chain reaction in biological membranes and lipoproteins (Leichtle et al. 2006).

Vitamin E is also the main antioxidant present in the LDL particle, making it important to investigate the interaction of nicotine and the vitamin E / HOCl system, given that HOCl is one of the factors leading to LDL oxidation. HOCl reacts primarily with the chromanol ring system of this vitamin (as described using Trolox, a water soluble analogue), leading to formation of a quinone, with a gradual absorbance rise in ultraviolet region (Ho et al. 2000, Pattison et al. 2003).

According to published reports, the reaction of tertiary amines with hypochlorous acid leads to formation of quaternary chlorammonium ions that dramatically enhance the chlorination of free (2'-deoxy) nucleosides (Masuda et al. 2001), salicylate and sorbate (Prutz 1998).

A recent study demonstrated that nicotine increase CD36 expression in macrophages (with consequent enhanced oxLDL uptake), contributing to the development of atherosclerosis (Zhou et al. 2013). This study demonstrates the important role of nicotine on LDL in reactive oxygen species generation systems, and their deleterious effect on the development of atherogenesis.

CONCLUSIONS

In conclusion, we have shown that nicotine enhances the oxidation of tryptophan (free or in LDL) by the MPO/H_2O_2/Cl^- system and the oxidation/ chlorination of its lipid components by HOCl directly. Several studies support the hypothesis that the risk of atherosclerosis is associated with the oxidative modification of LDL and there is diverse evidence that MPO and HOCl play a part in the development of atherosclerosis. The results presented here afford evidence on the biochemical effects of nicotine on components involved in the pathogenesis of cardiovascular diseases.

Abbreviations: LDL, low-density lipoprotein; ML, methyl linoleate; MPO, myeloperoxidase; PUFA, Polyunsaturated Fatty Acid; ROS, reactive oxygen species; TMB, 3,3',5,5'-tetramethylbenzidine.

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RESUMO

Nesse estudo, nós avaliamos o efeito da nicotina sobre a oxidação da LDL pelo sistema MPO/H_2O_2/Cl^- e o efeito do HOCl sobre a LDL e alguns de seus componentes, tais como metil linoleato, vitamina E e o aminoácido triptofano. Nicotina, em concentração micromolar, amplia a oxidação do triptofano, livre em solução ou presente na LDL. A nicotina promove a diminuição da formação de dienos conjugados e

consumo de oxigênio no sistema metil linoleato/HOCl, e houve evidência que sugere aumento na formação de clorohidrinas. O aumento da oxidação da vitamina E pelo HOCl também foi observada na presença de nicotina. Esses dados mostram que a interação da nicotina e HOCl pode promover alterações bioquímicas significativas na partícula de LDL e alguns de seus componentes envolvidos na patogênese de doenças cardiovasculares e de outras doenças.

Palavras-chave: nicotina, mieloperoxidase, ácido hipocloroso, lipoproteína de baixa densidade, metil linoleato, vitamina E, triptofano.

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