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Mechanisms and kinetics of heme iron nitrosylation in an *in vitro* gastro-intestinal model

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ABSTRACT

Fresh red meat and cured meat consumption increases the risk of gastro-intestinal cancers and it is strongly suspected that nitrosylheme is implicated by stimulating the endogenous production of mutagenic aldehydes and N-nitroso compounds. To investigate the extent of endogenous heme iron nitrosylation an experimental *in vitro* model that mimics the physicochemical conditions of the gastro-intestinal tract was used in association with a mathematical model of chemical reaction kinetics. The combined effect of pH (from 7.2 to 3.2) and myoglobin oxidation state was evaluated in the reaction of nitrite with heme iron, and the observed rate constants of the reactions were determined. Nitrosylation was optimal under mildly acidic conditions (pH 6.5–4.7). Up to 20% of myoglobin can be nitrosylated under gastro-intestinal conditions in this pH range. The effect of various antioxidants (from meat or vegetables) on the endogenous nitrosylation process was also tested.

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1. Introduction

Links between food nitrate and nitrite and health risk have been studied for more than 50 years. Many toxicity and epidemiological studies suggested that high dietary nitrate and nitrite intake is an aetiological factor in the development of some cancers, especially esophageal and gastro-intestinal cancers (Grosse et al., 2006; Keszei, Goldbohm, Schouten, Jakszyn, & van den Brandt, 2013). Nevertheless, the cancer hazard from nitrate/nitrite ingestion cannot be determined without considering other factors such as the presence of nitrosatable compounds (mainly found in meat) and antioxidants (mainly found in vegetables) (Grosse et al., 2006; Keszei et al., 2013). Dietary sources of nitrate and nitrite come primarily from vegetables and cured meats. In meat, nitrite is used to develop cured meat color and flavor and to prevent the growth of harmful bacteria (Pegg & Shahidi, 2000; Shahidi, Samaranayaka, & Pegg, 2014). Nitrate is not dangerous by itself but can be partially reduced to nitrite in the oral cavity (Van Maanen, van Geel, & Kleinjans, 1996). Due to their high level of nitrate vegetables contribute considerably (around 90%) to the global ingestion of nitrites in humans (Thomson, 2004).

The first risk of nitrite is due to its reaction with dietary secondary amines during food processing or gastrointestinal digestion to form nitrosamines, some of which are mutagenic. This risk has long been described (Bartsch & Montesano, 1984; Pegg & Shahidi, 2000) and it has been well documented in the literature. The second risk of nitrite is due to its reaction with the heme iron of myoglobin to form nitroso-myoglobin. Nitroso-myoglobin gives the dark red color characteristic of raw cured meats. During thermal processing globin denatures and detaches, leading to the formation of nitroso-ferrohemochrome which gives the pink color characteristic of cooked cured meats. These two pigments can release nitrosylheme during heating (Pegg & Shahidi, 2000) and digestion. The mutagenicity of nitrosylheme has recently been reported. Nitrosylheme could be involved in carcinogenesis via the production of mutagenic aldehydes (hydroxyhexanal and hydroxynonenal) formed under the free-radical process involving heme iron, and via the formation of various N-nitroso compounds (NOCs) which can react with DNA (Kuhnle & Bingham, 2007; Santarelli, Pierre, & Corpet, 2008; Santarelli et al., 2010). The endogenous formation of nitrosylheme is also strongly suspected in addition to the exogenous contribution. Indeed, it has been demonstrated that a red meat diet increased the level of nitrosylheme at ileal level and in the stools of volunteers, compared with a vegetarian diet (Kuhnle & Bingham, 2007; Lunn et al., 2007). Moreover, other studies have demonstrated that red meat, but not white meat, consumption can increase NOC excretion in feces (Bingham, Pignatelli, Pollock, Ellul, Malaveille, Gross, & O'Neill, 1996; Lunn et al., 2007) and the formation of NOC-specific alkylating DNA adducts in colonic cells (Lewin et al., 2006). In an *in vitro* digestion model, Van Hecke et al. (2014) reported that colonic digestion of beef resulted in higher

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concentration of a specific NOC-DNA adduct compared to chicken and pork. These authors reported that myoglobin levels in chicken and pork did not differ significantly while these two animal species had significant lower myoglobin levels compared to beef. All these studies strongly suggested the possible formation of nitrosylheme in the digestive tract through the reaction of heme iron of myoglobin (mainly provided by red meat) with nitrites (mainly provided by vegetables). This endogenous nitrosylation could explain the modest but significant association between red meat consumption and the risk of colorectal cancer (Larsson & Wolk, 2006; Norat, Lukanova, Ferrari, & Riboli, 2002). The International Agency for Research on Cancer has recently classified cured meats as carcinogenic for humans, while fresh red meats were only classified as probably carcinogenic for humans (Bouvard et al., 2015). The mechanisms and kinetics of heme iron nitrosylation in meat during the curing process have been widely studied. On the contrary, endogenous nitrosylation that could occur in human body is still poorly documented and there are no clearly demonstrated biological mechanisms that could explain the risk difference between cured and uncured meats.

It is, therefore, essential to provide new insights on the mechanisms and kinetics of endogenous heme iron nitrosylation and to better estimate its risk for humans. For this purpose, a new approach was used coupling an *in vitro* experimental model with a mathematical model of chemical reaction kinetics. The experimental model was composed of myoglobin (under different oxidation states) and nitrite, placed under the physicochemical conditions (pH, pO_2, ionic strength, temperature, and reducing conditions) of the digestive tract. To take into account the complexity of the diet and the interactions between the different nutrients, various antioxidants, in concentration representative of different meals, were then added to the model.

2. Materials and methods

2.1. Reagents

All the reagents used in this study were purchased from Sigma Aldrich France.

2.2. Composition of the reaction model

Heme iron nitrosylation was evaluated in a saline solution composed of $KCl/NaCl/CaCl_2$ (2/120/6 mM) with sodium phosphate 20 mM. Sodium nitrite and ascorbate were added to this solution at the final concentration of 3 mM, with pH adjusted at different values (7.2, 6.5, 5.7, 5.3, 4.7, 4, and 3.2). Myoglobin (40 μM) was added to the model in three forms: metmyoglobin, oxymyoglobin, or a mixture (1/1) of these two forms. Metmyoglobin (oxidized myoglobin with iron in ferric form, Mb (Fe^{3+})), the main form (98%) of commercial myoglobin (from equine skeletal muscle), was used as is. Oxymyoglobin (the oxygenated form of myoglobin with iron in ferrous form, Mb (Fe^{2+})-O_2) was prepared from the commercial metmyoglobin with the following protocol. Metmyogobin was reduced into deoxymyoglobin (Mb (Fe^{2+})) by incubation for one hour in an ice bath with ascorbate in large excess (100 mM) in the phosphate buffer previously described. The excess ascorbate was then eliminated by ultrafiltation in an *Amicon*® cuvette fitted with a polyethersulfone membrane with a cutoff at 10 kDa molecular weight (from Millipore®). During the ultrafiltration, agitation in air allowed the oxygenation of the reduced deoxymyoglobin into oxymyoglobin. Under these conditions, myoglobin finally reached 97 ± 3% in oxygenated form. It was not possible to start the reaction with oxymyoglobin at pH 3.2, due to the fast oxidation of heme iron. Therefore, only metmyoglobin was tested at this pH. The mixture (1/1) of these two forms was prepared just before measurement, leading to 50% of oxymyoglobin and 50% of metmyoglobin. The concentration of the whole myoglobin was evaluated by the method of Hornsey (1956) and its oxidation state was evaluated by that of Krzywicki (1979). Antioxidants were tested only at pH 5.3 with oxymyoglobin as initial pigment. All antioxidants: uric acid, carnosin, glutathione, trolox C (water-soluble analog of vitamin E), β carotene, natural polyphenols (caffeic acid and rutin) and butylated hydroxytoluene (BHT) were added at three different concentrations (16, 40, and 80 μM). Antioxidants were prepared as 100-fold stock solutions in the phosphate buffer (in case of uric acid, carnosin, glutathione) or in ethanol (in case of trolox C, β carotene, polyphenols, and BHT) before addition to the reaction medium.

After the addition of the different nitrosylation reagents, the samples were incubated at 37 °C in a dry bath incubator fitted with block heaters (Prolabo BT3) under agitation, and the kinetics were evaluated for 2 hours with the following sampling times: 0, 15, 30, 45, 60, and 120 minutes. The antioxidant effect was evaluated only after 60 minutes. The initial concentration of dissolved oxygen in the model was 0.22 ± 0.01 mM (measured with a luminescent dissolved oxygen probe; Hach HQ 440d).

2.3. Evaluation of heme iron nitrosylation

The heme iron nitrosylation was evaluated according to the method of Hornsey (1956) with slight modifications. The selective extraction of nitrosylheme from nitroso-myoglobin was achieved by adding 4 volumes of acetone to 1 volume of the reaction medium. After 20 s of vigorous agitation, the samples were filtered on syringe filters with 0.45 μm regenerated cellulose membranes (Interchim®, France). Under these conditions, no interference occurred with the heme of oxymyoglobin or metmyoglobin which cannot be extracted in acetone without the prior addition of concentrated HCl (Hornsey, 1956). Absorbance measurements of the filtrates were performed on a Jasco V-770 spectrometer. The level of nitrosylheme formed was evaluated by measuring the specific absorbance at 540 nm with an absorption coefficient of 11.3 mM^{-1} cm^{-1}, corresponding to the nitrosylheme in the acetone/water (80:20, v/v) mixture (Hornsey, 1956). In a recent publication (Yu, Jiao, Ma, & Sun, 2016) it was demonstrated that strongly acidic (pH < 2) or alkaline (pH > 8.6) conditions affected the 540 nm absorbance of nitrosylheme in the acetone/water (80:20, v/v) mixture, but in the pH range used in our study this absorbance was unchanged. All the measurements were performed against a blank composed of all the reagents, except nitrite, in the acetone/water (80:20, v/v) mixture.

2.4. Modeling of kinetics and calculation of the observed rate constants (k)

The kinetics were analyzed with a chemical reaction simulator described in our previous publication on N-nitrosation (de La Pomélie, Santé-Lhoutellier, & Gatellier, 2017). The system of differential equations corresponding to a chemical-kinetics mechanism was solved by the Runge Kutta method. With this simulator, a pseudo first-order reaction model was used to determine the observed rate constants (k). The observed first-order rate constants were varied to obtain the best adjustment between the simulated and the experimental levels of nitrosylheme. The simulations were optimized by reducing to the maximum the sum of the squared deviations between the experimental and calculated data. The observed first-order rate constants were expressed in s^{-1}. In the case of reversible reactions, the observed equilibrium constants K were calculated (K = k forward/k reverse).

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