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Oxidation and nitrosation of meat proteins under gastro-intestinal conditions: Consequences in terms of nutritional and health values of meat

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ABSTRACT

The chemical changes (oxidation/nitrosation) of meat proteins during digestion lead to a decrease in their nutritional value. Moreover, oxidized and nitrosated amino acids are suspected to promote various human pathologies. To investigate the mechanisms and the kinetics of these endogenous protein modifications, we used a dynamic artificial digestive system (DIDGI®) that mimics the physicochemical conditions of digestion. The combined effect of meat cooking and endogenous addition of ascorbate and nitrite was evaluated on protein oxidation (by measuring carbonyl groups), protein nitrosation (by measuring nitrosamines), and proteolysis. Considerable carbonylation was observed in the digestive tract, especially under the acidic conditions of the stomach. Nitrosamines, caused by ammonia oxidation, were formed in conditions in which no nitrite was added, although the addition of nitrite in the model significantly increased their levels. Meat cooking and nitrite addition significantly decreased protein digestion. The interactions between all the changes affecting the proteins are discussed.

1. Introduction

Meat and meat products are a good source of proteins for humans. These proteins are well balanced in amino acids and contain all the essential amino acids that humans cannot synthesize. Nevertheless, meat processing (storage, cooking, salting, and curing) can affect the chemical state of proteins by oxidation and, to a lesser extent, by nitrosation. These reactions initiated during meat processing can then develop during digestion. Indeed, the physicochemical conditions of the stomach such as oxygen pressure, low pH, and reducing conditions, favour the formation of reactive oxygen species like superoxide and hydroxyl radicals (Oueslati, de La Pomélie, Santé-Lhoutellier, & Gatellier, 2016), and nitrite derivatives like nitric oxide and nitrosonium ions (de La Pomélie, Santé-Lhoutellier, & Gatellier, 2017). Moreover, the digestive tract is characterized by the disintegration of solid foods and the enzymatic degradation of proteins, rendering amino acids more sensitive to these very reactive compounds (Kong & Singh, 2008). The carbonylation of basic amino acids, thiol oxidation, and aromatic hydroxylation are the main oxidative modifications generated by reactive oxygen species (Promeyrat, Daudin, & Gatellier, 2013). Amino acids containing free thiols and secondary amine groups are the target of nitrite derivatives, leading to the formation of nitroso-thiols (*S*-nitrosation) and nitrosamines (*N*-nitrosation), respectively (Goldstein & Czapski, 1996; de La Pomélie et al., 2017).

Oxidation and nitrosation negatively affect the nutritional value of meat products by modifying amino acids, some of which are essential amino acids. Amino acid oxidation is also the main cause of protein aggregation, leading to decreased digestibility, as observed *in vitro* by Santé-Lhoutellier, Aubry, & Gatellier, 2007. Besides this decrease in nutritional value, protein modifications may negatively impact human health. Indeed, oxidized amino acids have been linked to bladder cancer (Chung & Gadupudi, 2011) and irritable bowel disease (Xie et al., 2014). Oxidized amino acids may also contribute to malfunction, apoptosis and various diseases through use for *de novo* synthesis of proteins in the human body (Dunlop, Brunks, & Rodgers, 2011). Moreover, the reduced digestibility of oxidized proteins leads to the accumulation in the colon of non-hydrolyzed proteins that can be fermented by colonic flora into mutagenic products like phenols and cresols (Evenepoel et al., 1998). Regarding nitrosamines, most of them are mutagenic in humans (Pegg & Shahidi, 2000; Shephard, Schlatter, & Lutz, 1987) and nitroso-thiols are the precursors of the formation of mutagenic nitrosylheme in the gut (Kuhnle & Bingham, 2007). This gastro-intestinal reactivity could explain the modest but significant association between red meat consumption and the risk of cancer, thus leading the International Agency for Research on Cancer to classify red meat as probably carcinogenic for humans (group 2A) (Bouvard et al., 2015).

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The mechanisms and kinetics of protein changes during meat processing have been well documented for a long time. On the other hand, endogenous oxidation and nitrosation that could occur in the human body and their impacts on protein digestion are still poorly documented. Thus, the estimation of protein changes under gastro-intestinal conditions is of great interest for evaluating the nutritional and health qualities of meat products, and sensitive tools for studying this process are required.

In vivo studies are too complex and expensive to be used in screening experiments aimed at evaluating gastro-intestinal reactivity. Our team has recently conducted studies to characterize protein and lipid oxidation (Oueslati et al., 2016) and *N*-nitrosation (de La Pomélie et al., 2017), in oversimplified, mono-compartmental, static *in vitro* systems involving incubation at fixed pH. Such simplified static models can be useful to determine kinetic laws and reaction mechanisms but they cannot reproduce the dynamics and transient nature of the *in vivo* digestion process. To better reflect human gastro-intestinal conditions, a dynamic *in vitro* artificial digestive computer-controlled system (DIDGI®) was used in the present study. This system can reproduce the main digestion parameters (temperature, pH changes, and enzymatic secretions) with good reliability.

The study reported here comprised the use of this digestive system to investigate the effect of the endogenous addition of ascorbate, with or without the co-addition of nitrite, on the extent of protein carbonylation, *N*-nitrosation, and proteolysis during the digestion of raw and cooked bovine meat. The interactions between these different reactions were analysed and discussed and a global model of this endogenous chemistry was proposed.

2. Materials and methods

2.1. Reagents

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

2.2. Meat cooking

The experiment was carried out on bovine M. *semimembranosus* from Charolais heifer. To prevent inter-animal and muscle variabilities only one muscle from one animal was tested in this study. Muscle was aged in vacuum for 13 days at 4 °C. Muscle was then sliced into 1-cm-thick steaks (weight 50 ± 2 g). Steaks were cooked in vacuum bags by immersion in a water bath (Polystat CC3 from Huber) at 60 °C or 90 °C for 30 min. These temperatures were selected to cover a wide range of modes of cooking. After cooking, steaks were cooled in ice for 10 min. Meat was then minced with a meat grinder (Pro 2000 Excel from Kenwood) through 8-mm-diameter holes. The cooking juices were reincorporated in the minced meat to form *in vitro* food boluses. All the *in vitro* boluses were then stored at –80 °C until use.

2.3. In vitro dynamic digestion of meat

Digestion was performed in an *in vitro* dynamic system (DIDGI®; INRA, Paris, France). This system was recently presented by Ménard et al. (2014), who used it to simulate infant digestion of human milk. This system comprised three consecutive compartments representing the gastric, duodenal/jejunal and ileal parts. A Teflon stopper with 2-mm holes was placed between the stomach and the intestine to mimic the human pylorus. Each compartment was fitted with pH and temperature probes. Two pumps used to add HCl and $NaHCO_3$ regulated the pH in the gastric and duodenal/jejunal compartments, respectively. In the gastric compartment, two other pumps added pepsin and gastric lipase. The last pump added pancreatin in the duodenal/jejunal compartment. Bile was not added in the model because of the interference between bile pigments and nitrosamine absorbance. This dynamic *in vitro* system was controlled by StoRM® software (Guillemin et al., 2010), which allows regulation and monitoring of digestive parameters.

The parameters (kinetics of pH and enzyme flow rates) were chosen to closely mimic healthy adult digestion. The temperature was maintained constant at 37 °C. In the gastric compartment fasted conditions were based on 150 mL of simulated gastric fluid (KCl 6.9 mM, NaCl 47.2 mM, $CaCl_2$ 8 mM, KH_2PO_4 0.9 mM, $NaHCO_3$ 25 mM, $MgCl_2$ 0.1 mM, $(NH_4)_2CO_3$ 0.5 mM) adjusted to pH 2, into which pepsin (15602U) was added. The composition of this simulated gastric fluid was taken from the international consensus paper by Minekus et al. (2014), which proposed a standardized digestion method based on human physiologically relevant conditions. After the addition of 20 g of *in vitro* food bolus (from raw or cooked meat), the pH rose to 5.5. The pH value in the gastric compartment followed a linear regression and returned to 2 in 120 min. Regarding the intestine, the pH varied from 6.5 to 7 in the duodenal/jejunal compartment and remained stable at 7 in the ileal part. Gastric enzymes were dissolved in the simulated gastric fluid at pH 2 while pancreatin was dissolved in distilled water. Enzyme flow rates were from 520 U/min for pepsin, 20 U/min for gastric lipase, and 1 U/min for pancreatin. The transit time in the stomach and in the intestine followed an exponential pattern fitted by the mathematical model described by Elashoff, Reedy, and Meyer (1982). The model is described by the equation $f = 2^{-(t/t_{1/2})\beta}$, where f is the remaining fraction in the compartment, t is the emptying time, $t_{1/2}$ is the emptying half-time ($t_{1/2}$ = 95 min. in the stomach and 250 min in the duodenum/jejunum), and β is the coefficient of the delivery curve (β = 2 in stomach and 2.5 in duodenum/jejunum). With this model, gastric, duodenal/jejunal and ileal emptying began after 12 min, 41, and 41 min. respectively.

Digestion took place in the presence of a mixture of sodium nitrite (1 mM) and sodium ascorbate (1 mM), or in the presence of ascorbate (1 mM) only, supplied as a powder form in the initial simulated gastric fluid. In each compartment the food bolus was maintained under constant agitation with a rotating blade actuated by a motor (rotation speed = 60 rpm). Samples (5 mL) were collected at different times after the beginning of digestion, namely: 0, 20, 60, and 120 min in the gastric compartment (G), 60, 120, and 150 min in the duodenal/jejunal compartment (D/J), and 120, 150, and 210 min in the ileal compartment (I). *N*-Nitrosation measurements were performed immediately after sampling, while several aliquots were stored at −80 °C for the other measurements. Digestion experiments were performed in triplicate.

2.4. Presentation of the results

In dynamic digestive conditions, the expression of the results is debatable. Indeed, the concentration of each product depends on biochemical reactivity in the digestive tract and on food bolus dilution due to the addition of various fluids, emptying, and sampling. In some cases, the dilution effects are so important that they can render difficult the analysis and interpretation of results. This is generally the case at the end of the digestion. As the present study focused on reactivity, we decided to eliminate the dilution effect by multiplying all the concentrations with dilution correction factors. These correction factors were estimated by the StoRM® software, which was used to manage all the fluid additions and the emptying speed. The correction factors were determined at the different measurement times in the gastric (G20: × 1.11, G60: × 1.30, G120: × 1.61), duodenal/jejunal (D/J60: × 1.69, D/J120: × 2.90, D/J150: × 3.42), and ileal (I120: × 2.90, I150: × 3.42, I210: × 3.86) compartments respectively. The corrected concentrations must be considered as theoretical concentrations allowing arguing in constant volume. We are well aware that the corrected concentrations do not reflect the true concentrations in the model but from our point of view, they are the best representation of the gastro-intestinal reactivity. Moreover, from these corrected concentrations it would be easy to extrapolate to the true concentrations under any conditions at all, as long as the dilution factors are