Inulin attenuates atherosclerosis in apolipoprotein E-deficient mice

Marie-Hélène Rault-Nania[1], Elyett Gueux[1], Céline Demougeot[2], Christian Demigné[1], Edmond Rock[1] and Andrzej Mazur[1]*

[1]*Equipe Stress Métabolique et Micronutriments, Unité de Nutrition Humaine UMR1019, INRA, Theix, Centre de Recherche en Nutrition Humaine d'Auvergne, 63122 Saint-Genès-Champanelle, France*
[2]*Laboratoire de Physiologie et Pharmacologie – Nutrition Préventive Expérimentale, Faculté de Médecine et de Pharmacie, Besançon, France*

Effects of different inulin-type fructan fractions were studied on atherosclerotic plaque formation in male apo E-deficient mice. Thirty-two mice were randomly divided into four groups and received either a semi-purified sucrose-based diet (control group), or diets in which sucrose was replaced in part by various inulin-type fructans (10 g/100 g): long-chain inulin, oligofructose, or an oligofructose-enriched inulin for 16 weeks. The presence of atherosclerotic plaques was assessed by histomorphometry in the aortic sinus. The apo E-deficient mice fed long-chain inulin or an oligofructose-enriched inulin had about 35 % and 25 % less atherosclerotic lesion area compared with the control group, respectively. Feeding long-chain inulin significantly reduced plasma cholesterol concentrations ($P<0.001$), and the three inulin-type fructans reduced triacylglycerol (TAG) concentrations compared with the control group ($P<0.001$). Both the long-chain inulin and an oligofructose-enriched inulin significantly lowered hepatic cholesterol concentrations compared with the control diet ($P<0.05$). Hepatic TAG concentrations were significantly lower in all three groups fed the fructan-supplemented diets *v.* the control group ($P<0.0001$). The results of the present study suggest that inhibition of atherosclerotic plaque formation is more potent in the presence of long-chain inulin, either alone or in combination with oligofructose (an oligofructose-enriched inulin), and that this probably is related to changes in lipid metabolism.

Inulin: Oligofructose: Apolipoprotein E-deficient mice: Atherosclerosis

Atherosclerosis is an important risk factor of CVD (Charakida *et al.* 2006), which is one of the major causes of mortality in Western countries (Steinberg *et al.* 2003). Hyperlipidaemia, often associated with inappropriate food choices (Cordain *et al.* 2005), is well recognised as a risk factor for the development of atherosclerosis (Wouters *et al.* 2005). In order to promote the consumption of complex carbohydrates, which are naturally present in fruits and vegetables (Roberfroid, 2002), various food items are nowadays supplemented with these. In addition, inulin-type fructans can also be used as sugar and/or fat substitutes in a variety of food applications such as milk products, baked goods, fruit preparations and confectionery (Kaur & Gupta, 2002). Inulin-type fructans are non-digestible oligosaccharides made up of fructose monomers (some with a terminal glucose) and are classified as soluble dietary fibre (Cherbut, 2002). Previous studies in animals and human subjects have found a hypolipidaemic effect when inulin was supplemented to the diet (Beylot, 2005). However, inulin and oligofructose also have other effects that might contribute to the anti-atherogenic action. Busserolles *et al.* (2003) showed that oligofructose is protective against the pro-oxidative effects of fructose-rich diets in rats. In addition, endproducts of dietary fibre fermentation, such as SCFA, can

modulate the expression of multiple genes involved in the process of atherosclerosis (Ranganna *et al.* 2000). Based on these data it can be hypothesised that the addition of inulin-type fructans to diets may reduce the atherosclerosis process, similar to the anti-atherosclerotic effect described for some dietary fibres (Wu *et al.* 2003).

In the present study, we investigated the effects of different fractions of inulin-type fructans in an apo E-deficient atherosclerosis mouse model, which is widely used to study the atherogenic process (Meir & Leitersdorf, 2004). The mouse model corresponds to familial dysbetalipoproteinaemia and the animals develop lesions even on a standard low-fat diet, the earliest lesions appearing in the aortic valve (Napoli *et al.* 2000). In the present study we assessed the anti-atherogenic effects of inulin-type fructans with different degrees of polymerisation (DP), for example, oligofructose (short chain) and long-chain inulin. Shorter inulin-type chains are rapidly and selectively fermented by the colonic flora leading to a high increase in bifidobacteria in the proximal colon. Long-chain inulin is fermented at a lower rate, inducing its prebiotic effect towards the more distal (descending) parts of the colon (Roberfroid, 2005). The oligofructose-enriched inulin is a combination of both inulin types and

therefore it exerts a sustained prebiotic effect throughout the entire colon.

Materials and methods

Animals and diets

Pairs of homozygous apo E-deficient mice were provided by Jackson Laboratories (Charles River Laboratories, L'Arbresle, France). The males for the present study were obtained through interbreeding these homozygous mice at the Institute animal facility (Unité de Nutrition Expérimentale, INRA, Theix, France). They were housed in groups of four in wire-bottomed cages in a temperature-controlled room (22 ± 0.8°C) with a 12 h light–dark cycle and a relative humidity of 55 ± 10%. The mice were maintained and handled according to the recommendations of the INRA Ethics Committee – decree no. 87–848. The mean body weight of mice at the start of the experiment was 20·5 (SE 0·5) g. All mice were fed a powdered purified diet, based on the AIN-93G recommendations, in which all carbohydrates were supplied as sucrose (Table 1). At 9 weeks of age, mice were divided into four groups (eight mice per group). One group received a sucrose-based diet (control group), and the three other groups received diets in which sucrose was substituted in part with various inulin-type fructans (6·3 % (w/w) during the 3-week adaptation period and 10 % (w/w) during the following 13 weeks). Table 1 provides the composition of the diets (given as wt/wt %). Food and distilled water were provided *ad libitum*. The inulin-type fructans studied (Beneo™) were obtained from Orafti (a division of Raffinerie Tirlemontoise, Tienen, Belgium). Oligofructose (Beneo™ P95) is obtained by partial enzymic hydrolysis of native inulin, extracted from the chicory root, with a DP ranging from 2 to 8 (average DP 4). Long-chain inulin (Beneo™ HP) is obtained from the physical removal of the lower-DP fraction present in native inulin and has a DP from 10 to 65 (average DP 25). Beneo™ Synergy1 is an oligofructose-enriched inulin and is made up of a mixture of oligofructose and long-chain inulin.

Mice were examined for clinical appearance twice per week (when they were weighed and when the litter in the cages was changed). The systolic blood pressure was measured at the 15th week by a tail-cuff method with a BP 2000 Blood Pressure Analysis System (Visitech System, Apex, NC, USA). The

mice were trained for blood pressure measurements to reduce stress-inducible changes in this parameter and the presented results correspond to the third measurement.

At the end of the experiment, mice were killed under pentobarbital anaesthesia. Blood was collected from the abdominal aorta into heparinised tubes. Plasma was prepared by low-speed centrifugation. The plasma samples were stored at −80°C. The organs were washed with heparinised physiological saline in the systemic pathway. The heart (with aorta) and the liver of each mouse were harvested in liquid N_2 and stored at −80°C until analysis. The whole caecum and the caecum contents were weighed individually for each mouse.

Plasma and hepatic lipids

Plasma total cholesterol and triacylglycerol (TAG) concentrations were measured using enzymic assays (Biomérieux, Marcy-l'Etoile, France) as described previously (Mazur *et al.* 1990). Liver samples were homogenised in KCl (9 g/l) with a Polytron homogeniser (Kinermatica GmbH, Lucerne, Switzerland) and lipids were chloroform–methanol (2:1, v/v) extracted under overnight agitation. The chloroform phase was recovered after centrifugation and evaporated under dry air. TAG from the lipid residue were saponified with 0·5 M-KOH-ethanol at 70°C for 30 min followed by the addition of 0·15 M-$MgSO_4$ to neutralise the mixture. After centrifugation (2000 **g**; 5 min) the concentrations of glycerol in the supernatant fractions were determined. The cholesterol in the lipid residue was dissolved with isopropanol. Total cholesterol and TAG levels were determined by enzymic assay (Biomérieux). Absorbance at 492 nm was measured in a spectrophotometer (Uvikon 941 plus series; Kontron instruments, St Quentin en Yvelines, France). A control serum (Biomérieux) was processed in parallel to verify the accuracy of the plasma and tissue lipid analyses.

Assessment of atherosclerosis

Quantification of atherosclerotic lesions was done by calculating the lipid deposition size in the aortic sinus as previously described (Nicoletti *et al.* 1998). Briefly, the peripheral fat of the upper aorta was removed and the thoracic and abdominal aortas were discarded. The heart with the aortic arch was dissected under a stereo microscope and frozen (in liquid N_2) in optimal cutting temperature embedding medium for serial cryo-sectioning covering 400 μm of the aorta root. The heart (stored at −80°C) was cut in a microtome (Frigocut 2800 E; Reichert-Jung, Nussloch, Germany) at −20°C. Sections (10 μm thick) were collected at every 100 μm throughout the aortic sinus (300 μm of the distal portion) and analysed. The distal portion of the aortic sinus was recognised by the three valve cusps that are the junction of the aorta and the heart. Cryostat sections were evaluated for fatty streak lesions after staining with Oil red O and counterstaining with haematoxylin. Each section was evaluated for Oil red O staining area by capturing images directly from a colour camera (Sony XC-71P CCD RGB, Kenmore, WA, USA) attached to an Olympus light microscope (Reichert-Jung Polyvar, Vienna, Austria) and displaying them on a RGB monitor by using Visilog software (Noesis, Crolles, France). Image analysis was carried out using the ImageJ free software (http://rsb.info.nih.gov/ij/). In order to reduce errors induced by sectioning angle, results were

Table 1. Formulation of the diets†

Ingredient	Control	Inulin-type fructans*
Sucrose (%)	63	53
Inulin-type fructan (%)	0	10
Casein (%)	20	20
Maize oil (%)	7	7
Cellulose (%)	5	5
Mineral mix AIN-93 G (%)	3·5	3·5
Vitamin mix AIN-93 G (%)	1	1
L-Cystine (%)	0·3	0·3
Choline bitartrate (%)	0·25	0·25
tert-Butylhydroquinone (%)	0·0014	0·0014

* The inulin-type fructans used were oligofructose, long-chain inulin or an oligofructose-enriched inulin.

† For details of diets and procedures, see p. 841.

expressed as the percentage of the cross-sectional vessel area stained with Oil red O.

Statistical analysis

Results are expressed as mean values with their standard errors. Data were analysed by one-way ANOVA coupled with the Student–Newman–Keuls multiple comparison test (GraphPad Instat; GraphPad Software Inc., San Diego, CA, USA), except for the analysis of the lesions areas for which we used a two-way ANOVA (studied group and the aortic section effect) (REGWG test; Statview; SAS Institute Inc., Cary, NC, USA). Differences were considered significant if $P<0.05$. The data were transformed when the SD between groups was significantly different.

Results

Animals

There were no significant differences in final body masses and in the relative liver mass among groups (Table 2). However, all fructan-fed groups had significantly heavier (about 2·5–3 times) caecal weight (whole caecum, caecum contents and caecum wall) when compared with the control group ($P<0.0001$) (Table 2). The three fructan-enriched diets did not significantly modify the systolic blood pressure compared with the control diet after 15 weeks on the experimental diets (Table 2).

Plasma and hepatic lipid concentrations

As shown in Table 3, the apo E knockout mice showed marked hypercholesterolaemia (13·56 (SE 0·73) mmol/l in the control diet-fed group). This is severe hypercholesterolaemia; the level is about seven times higher than in C57/Bl6J mice (1·85 mmol/l data from Mouse Phenome Database; Jackson Laboratory, Bar Harbor, ME, USA; http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home). Feeding the mice inulin significantly lowered plasma total cholesterol concentrations compared with the control diet-fed group. Oligofructose and Synergy1 had no significant effect on the plasma cholesterol when compared with the controls ($P<0.001$). Plasma TAG concentrations were

significantly lower both in the inulin and oligofructose groups compared with the control group ($P<0.01$) (Table 3).

Supplementing the diet with inulin and Synergy1 significantly lowered hepatic cholesterol concentrations compared with the control diet ($P<0.05$). This effect was not significantly different for the oligofructose group. All three groups fed the fructan-enriched diets presented an approximately 2-fold reduction in hepatic TAG concentrations when compared with the control group ($P<0.0001$) (Table 3).

Atherosclerotic lesions in the aortic sinus

The two-way ANOVA analysis indicated that there was both an effect of the diet and the aortic section ($P<0.0004$), but there was no interaction between diet and aortic section ($P>0.05$). The REGWQ test (Statview; SAS Institute Inc.) showed that the percentage lesion area in the mice fed inulin was significantly lower compared with both the control and the oligofructose-fed groups ($P<0.01$). Furthermore, in the rats fed Synergy1 (Orafti), the percentage of lesion was significantly lower compared with the control group ($P<0.01$). The mean lesioned areas were 35 and 25 % lower in the inulin- and Synergy1-fed groups than in the control group, respectively (Table 3). The representative pictures from aorta sections of the control and inulin-rich diet-fed mice are presented in Fig. 1. It is noticeable that inulin feeding results in the reduction of red-stained lipid deposits when compared with the control.

Discussion

Inulin-type fructans of various chain lengths are fermented at different parts of the large intestine, as a function of their DP, and this in turn may have different metabolic consequences. In the present study, the effect of different inulin-type fructans on atherosclerosis lesion formation in apo E-deficient mice was assessed.

The present study shows that diets containing long-chain inulin, either alone or in combination with oligofructose, significantly reduce the area of the atherosclerotic plaque in the aortic sinus when compared with control diets. All the groups studied exhibited hypercholesterolaemia. This is in agreement with the apo E-deficient mice model, in which

Table 2. Body weight, relative liver weight, caecum weight and blood pressure in male homozygous apolipoprotein E-deficient mice after 4 months on the control or inulin-type fructans-supplemented diets* ‡

(Mean values with their standard errors for eight mice per group)

	Control		Oligofructose		Long-chain inulin		Synergy1		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA (*P*)
Mice weight (g)	23·93[a]	0·56	28·25[a]	0·43	28·27[a]	0·59	29·15[a]	0·32	NS
Blood pressure (mmHg)†	110[a]	4	112[a]	2	108[a]	2	112[a]	3	NS
Relative liver weight (g/100 g body weight)	5·99[a]	0·16	5·35[a]	0·10	5·65[a]	0·34	5·61[a]	0·12	NS
Whole caecum weight (g)	0·21[a]	0·03	0·74[b]	0·06	0·62[b]	0·05	0·81[b]	0·07	<0·0001
Caecum content weight (g)	0·11[a]	0·01	0·48[b]	0·06	0·37[b]	0·04	0·53[b]	0·06	<0·0001
Wall caecum weight (g)	0·09[a]	0·02	0·26[b]	0·01	0·24[b]	0·01	0·27[b]	0·02	<0·0001

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

* The inulin-type fructans were oligofructose, long-chain inulin or an oligofructose-enriched inulin (Synergy1; for details, see p. 841 of proofs).

† Blood pressure measurement after week 15.

‡ For details of diets and procedures, see p. 841.

Table 3. Plasma and liver lipid concentrations and mean atherosclerosis lesioned area in male homozygous apolipoprotein E-deficient mice after 4 months on the control or inulin-type fructans-supplemented diets* †

(Mean values with their standard errors for eight mice per group)

	Control		Oligofructose		Inulin		Synergy1		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA (P)
Plasma cholesterol (mmol/l)	13·56[a]	0·73	11·55[a]	0·45	9·47[b]	0·41	14·38[a]	1·21	0·0003
Plasma triacylglycerol (mmol/l)	1·33[a]	0·15	0·68[b]	0·08	0·66[b]	0·16	1·00[a,b]	0·17	0·0071
Hepatic cholesterol (mg/g)	3·19[a]	0·15	2·71[a,b]	0·18	2·45[b]	0·20	2·47[b]	0·15	0·0166
Hepatic triacylglycerol (mg/g)	53·38[a]	4·97	27·73[b]	4·78	20·03[b]	2·53	23·87[b]	4·86	<0·0001
Mean atherosclerosis lesioned area (%)	14·01[a]	1·14	12·44[ab]	0·96	9·16[c]	0·77	10·45[bc]	1·07	0·0004

[a,b,c] Mean values within a column with unlike superscript letters were significantly different (P<0·05).

* The inulin-type fructans were oligofructose, long-chain inulin or an oligofructose-enriched inulin (Synergy1; for details, see p. 841 of proofs).

† For details of diets and procedures, see p. 841 of proofs.

chylomicron and VLDL remnants accumulate in the blood as a result of a defect in their clearance by the liver (Meir & Leitersdorf, 2004). Long-chain inulin lowered plasma cholesterol and TAG concentrations compared with the control group. Previous work by Mortensen *et al.* (2002) showed a (non-significant) tendency in low-density-receptor-knockout-deficient mice, fed inulin, to reduce the atherosclerotic process. The more potent effect of inulin in the present study could be explained by the model used, which is characterised by severe hypercholesterolaemia.

Consistent with other studies, the present data clearly support the hypocholesterolaemic (Mortensen *et al.* 2002) and hypotriacylglycerolaemic (Beylot, 2005) effects of inulin. This hypolipidaemic effect undoubtedly reflects reduced secretion of VLDL particles by the liver resulting from an inhibition of *de novo* fatty acid synthesis (Williams, 1999). Hepatic cholesterol and TAG contents are high in apo E-deficient mice compared with C57Bl/6J mice (Kuipers *et al.* 1996; Xia *et al.* 2003).

Diets enriched with long-chain inulin or Synergy1 both significantly reduced hepatic cholesterol contents. It could be hypothesised that the hypocholesterolaemic effect of inulin is due, at least in part, to the fact that inulin inhibited cholesterol synthesis by propionic acid (Williams, 1999). In the present study, intake of inulin-type fructans was associated with a significant increase in the weight of the caecum in all three groups when compared with the control group. This is consistent with published data by Zdunczyk *et al.* (2004) demonstrating an increase in the weight of the caecum with 8 % of inulin.

Indeed, inulin fermentation resulted in production of propionic acid, a major component among the SCFA products (Kim & Shin, 1998). The decrease in hepatic TAG content by the three complex carbohydrate-rich diets is probably the result of a decrease in their synthesis (Delzenne & Kok, 1999). This hypotriacylglycerolaemic effect could also affect the plasma cholesterol concentration because of the fact that TAG-rich particles, especially in apo E-deficient mice, are rich in cholesterol.

The pathogenesis of atherosclerosis is a complex process that depends on various factors (Tripathi *et al.* 2005). According to the present results, it seems that inulin reduced the atherosclerotic plaques mainly by modifying lipid metabolism. However, other mechanisms could also be involved. Busserolles *et al.* (2003) showed the pro-oxidative effects of sugar and that substitution of fructose by oligofructose reduced heart lipid oxidation. Thus, the substitution of simple carbohydrates for complex carbohydrates could contribute to the anti-atherogenic effect via mechanisms reducing oxidative stress. The specific action of SCFA should also be considered because they are known to modulate the expression of multiple genes involved in the pathogenesis of atherosclerosis, in addition to genes involved in lipid synthesis (Ranganna *et al.* 2000).

The present study demonstrates that long-chain inulin in the diet reduces the development of atherosclerotic plaque. Based on the present results, we hypothesise that inulin acts mainly by modulating lipid metabolism, but future studies will
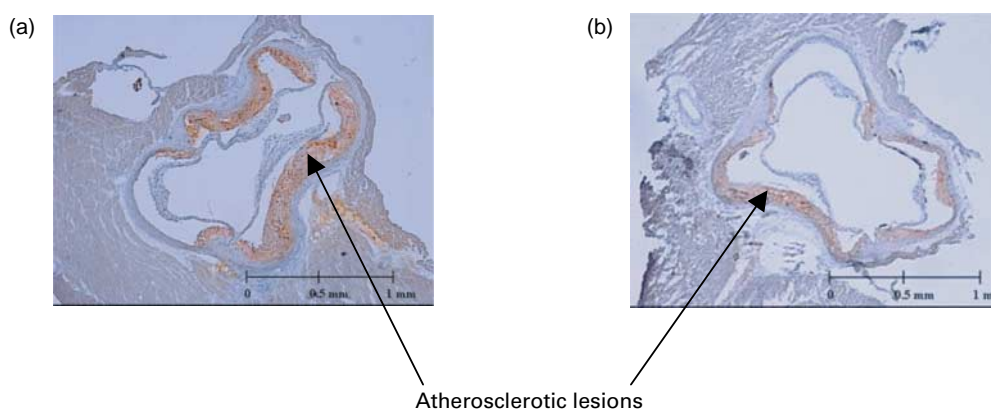

Atherosclerotic lesions

Fig. 1. Sections of aortic sinus (at 400 μm) stained by Oil red O from homozygous apo E-deficient mice fed a control sucrose-based diet (a) or an inulin-rich diet (b) for 16 weeks. For details of diets and procedures, see p. 841.

determine the contribution of the different mechanisms to the anti-atherogenic action of inulin.

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