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Antiatherogenic properties of lipid fractions of raw and fried fish

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Abstract

Numerous epidemiological studies have established the protective role of fish with respect to cardiovascular diseases. Platelet activation, thrombosis and inflammation are crucial phases of atheromatic plaque formation and platelet-activating factor (PAF) is a common mediator of these events which plays a pivotal role in atherogenesis. The presence of various lipids with PAF-like activity or PAF antagonists in various foodstuffs has been reported and may prove a valuable index of their nutritional value. Under this perspective the aggregatory properties of lipid fractions obtained from six of the most widely consumed fish (rainbow trout, golden trout, sea bass, herring, coley and plaice) were studied in either raw or fried samples. The total lipids were extracted and separated into neutral and polar lipid fractions. All fractions were tested for their ability to aggregate platelets or inhibit PAF-induced aggregation. The aggregatory properties of lipid fractions were dependent on the fish species. Frying led to fractions of reduced aggregatory activity probably due to the absorption of the frying medium, which contains PAF inhibitors. The aggregatory properties of total lipids were mainly attributed to polar lipids while the PAF antagonistic activities were attributed to neutral lipids. The biological activities of such fractions with respect to their aggregatory properties may explain, in part, the protective role of fish against cardiovascular diseases and determine their nutritional value.

Keywords: Fish; Lipids; Platelet; Aggregation; Frying; Platelet-activating factor; Antagonists

1. Introduction

Several epidemiological studies have demonstrated the protective role of fish and fish oil consumption against coronary heart diseases (Kris-Etherton, Harris, & Appel, 2003). The nutritional benefits of fish consumption were mainly attributed to the effects of ω − 3 polyunsaturated fatty acids, which are thought to have several potentially cardioprotective actions (Din, Newby, & Flapan, 2004). Activation of platelets and thrombosis are crucial events in atheromatic plaque formation and they have become a common therapeutic target in coronary syndromes (Di Minno, Tufano, Garofano, & Di Minno, 2002; Melandri et al., 2002). In Greenland Eskimos, the high intake of marine food is associated with low incidence of myocardial infarction along with a lower platelet count, inhibition of platelet aggregation and prolonged bleeding time (Dyerberg, 1989). However, the effects of ω − 3 fatty acids on platelet function and thrombosis are controversial, suggesting that other substances, apart from ω − 3 fatty acids, may be responsible for the antithrombotic properties of marine fish (Kristensen, Iversen, & Schmidt, 2001; Mori, Beilin, Burke, Morris, & Ritchie, 1997).

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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent inflammatory mediator implicated in the mechanism of many pathophysiological conditions in humans. It is among the strongest endogenous agonists of platelet aggregation (Demopoulos, Pinckard, & Hanahan, 1979; Prescott, Zimmerman, Stafforini, & McIntyre, 2000). Many experimental data, recently reviewed (Demopoulos, Karantonis, & Antonopoulou, 2003), implicate that PAF is a crucial mediator of atherogenesis. In this perspective, the presence of PAF-like activity molecules or PAF antagonists in various foodstuffs is very important in terms of nutritional value. Previous studies from our laboratories have shown that lipid fractions obtained from traditional foods of the Mediterranean diet, such as olive oil (Karantonis, Antonopoulou, & Demopoulos, 2002), wine (Fragopoulou, Antonopoulou, & Demopoulos, 2002), honey (Koussissis et al., 1994), milk and yogurt (Antonopoulou, Semidalas, Koussissis, & Demopoulos, 1996), exert both PAF-like and anti-PAF activities in washed rabbit platelets and the structures of some of these antagonists were identified. In an analogous study, gangliosides with a PAF-like activity as well as other PAF-like agonists and PAF antagonists were identified in mackerel (Scomber scombrus) (Rementzis, Antonopoulou, & Demopoulos, 1997; Rementzis, Antonopoulou, Argyropoulos, & Demopoulos, 1996).

Since most fish species are consumed cooked, the nutritional value of the final cooked product is of major importance for human health. The culinary processes can alter significantly the content, the composition and the biological activity of the fish lipids. Frying, which is commonly utilized for fish cooking, leads to an increase in the fat content of the fish fillet (Aro et al., 2000; Candela, Astiasaran, & Bello, 1997), extensive lipid exchanges between the fish and the frying medium (Sebedio, Ratnayake, Ackman, & Prevost, 1993) along with the production of oxidized and polymerized lipid products (Kubow, 1992; Skog, Johansson, & Jagerstad, 1998). In order to study the effect of frying on the biological activity (especially platelet activation) of fish lipid fractions, we previously studied the aggregatory activity of raw and fried cod lipids and we found that there is a range of PAF-like and anti-PAF activities in lipid fractions of fresh cod whereas in fried cod, anti-PAF activities were mainly observed (Panayiotou et al., 2000).

In this study, the lipid fractions of six of the most commonly consumed fish were tested for their ability to induce washed rabbit platelet aggregation or antagonize the PAF-induced platelet aggregation. The biological activity of the same lipid fractions was tested after frying of the fish in order to identify whether frying alters the nutritional value of the consumed product in terms of platelet aggregation. The fish species selected were rainbow trout (*Oncorhyncus mykiss*), golden trout (*Onchorhyncus aguabonita*), haddock (*Melanogrammus aeglefinus*), coley (*Pollachius virens*), plaice (*pleuronectes platessus*) and sea bass (*Dicentrarchus labrax*).

2. Materials and methods

2.1. Reagents and instrumentation

All reagents and solvents were of analytical grade and supplied by Merck (Darmstadt, Germany). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (Demopoulos et al., 1979). Platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA).

2.2. Fish samples

Six different species of fresh fish, namely rainbow trout (*Oncorhyncus mykiss*), golden trout (*Onchorhyncus aguabonita*), sea bass (*Dicentrarchus labrax*), haddock (*Melanogrammus aeglefinus*), coley (*Pollachius virens*) and plaice (*pleuronectes platessus*) were purchased from the local market. The first three species (rainbow trout, golden trout and sea bass) were farmed while the haddock, coley and plaice had been recently caught from the open sea and stored on chopped ice. The fish were manually filleted and each fish fillet was cut into three similar pieces. These pieces (approximately 50 g each) were either chopped prior to homogenization and used as fresh samples or fried in a Kenwood deep fryer with sunflower oil at 170 °C for 7 min. The fried pieces were allowed to cool and dried on paper. The skin of the fillets was then removed and these samples were composited and chopped prior to homogenization. For each run, three pieces from three different fillets were pooled and analyzed.

2.3. Lipid fractions of fish samples

Total lipids (TL) were extracted according to the Bligh and Dyer method (Bligh & Dyer, 1959). The 1/10 of the TL fraction was weighed and stored at −20 °C while the rest of it was further separated into neutral lipids (NL) and polar lipids (PL) by counter-current distribution (Galanos & Kapoulas, 1965). The NL and PL fractions were weighted and stored at −20 °C for further analysis.

2.4. Platelet aggregation assay

Total lipids, neutral lipids and polar lipids fractions were tested for their biological activity against washed

rabbit platelets as described previously (Demopoulos et al., 1979). Briefly, PAF and the examined samples were dissolved in a solution of 2.5 mg of bovine serum albumin (BSA) per ml of saline. Various concentrations of the examined sample were added into the aggregometer cuvette and the aggregation induced by the sample was measured as the percentage of the maximum reversible aggregation. The aggregatory activity of the sample was expressed as the amount of the sample in μg that is able to induce 50% of the maximum reversible aggregation of the respective sample. This value is defined as EC_{50}, namely 50% efficient concentration. In order to study the inhibitory activities of the lipid fractions, platelets were preincubated with the samples for 1 min prior to the addition of PAF (2.5×10^{-11} M, final concentration in the cuvette). The platelet aggregation induced by PAF was measured as PAF-induced aggregation before (considered as 0% inhibition) and after the addition of various concentrations of the examined sample. Consequently, the plot of percent inhibition versus different concentrations of the sample was constructed and from this plot the concentration of the sample that inhibited 50% PAF-induced aggregation (IC_{50}) was calculated. The IC_{50} values are expressed as μg of the sample.

3. Results and discussion

3.1. Total lipids, neutral lipids and polar lipid fractions of fresh and fried fish

Six fish species, widely consumed throughout the world, were examined for the platelet activating properties of their lipid fractions. Two samples were used for each fish, one of raw and one of fried flesh. The extraction of total lipids (TL) from fresh and fried fish samples along with their separation to neutral lipids (NL) and polar lipids (PL) was carried out by the counter current distribution. The extraction of TL was carried out by the method of Bligh and Dyer (Bligh & Dyer, 1959), which had been satisfactorily used for the extraction of mackerel lipids previously (Rementzis et al., 1997). We used distilled water instead of saline in the extraction solvent in order to achieve a better recovery of gangliosides, a lipid class with possible PAF or anti-PAF activity, in the chloroform phase. An amount (1/10) of total lipids (TL) was stored in −20 °C in order to test its biological activity while the rest was separated into neutral and polar lipids by counter current distribution chromatography (Galanos & Kapoulas, 1965). This method allows excellent recovery of polar lipids from neutral sources. By this procedure the total polar lipid fraction contained glyco- and phospholipids. The same procedure was followed for the fried fish samples, too. The amount of TL, NL, PL in g kg^{-1} of fish flesh is shown in Table 1. As it can be seen, the levels of polar lipids in the raw flesh are higher than the levels of neutral lipids. Frying resulted in an increase of the levels of total lipids; the lowest increase was found for the golden trout (1.5-fold) and the highest was found for the coley (8-fold). The increase of total lipids is attributed to a significant increase of neutral lipids (from 3.2-fold for golden trout to 25.9-fold for coley). On the other hand, frying led to a reduction of the amount of polar lipids (from 17% for golden trout to 50.2% for Coley). The higher amounts of fried samples neutral lipids were most probably due to the absorption of neutral lipids from the frying medium (sunflower oil) while the reduction of polar lipids could be explained by various reasons such as hydrolysis, which can be favored by the frying conditions or by extraction to the frying medium.

Table 1
Contents (g kg^{-1}) of total lipids (TL), polar lipids (PL) and neutral lipids (NL) in the flesh of raw and fried fish species

Fish species	Processing	TL	PL	NL
Golden trout	Raw	57.84 ± 6.32	31.04 ± 7.56	17.00 ± 0.96
	Fried	85.30 ± 10.45	25.52 ± 6.28	54.22 ± 6.32
Coley	Raw	10.67 ± 1.78	5.83 ± 0.34	2.83 ± 0.76
	Fried	85.21 ± 8.56	2.94 ± 0.18	73.32 ± 5.23
Sea-bass	Raw	30.67 ± 2.45	16.80 ± 2.23	11.19 ± 1.48
	Fried	82.82 ± 12.34	9.32 ± 0.89	76.82 ± 6.95
Plaice	Raw	23.56 ± 1.78	13.27 ± 0.96	6.55 ± 0.41
	Fried	59.42 ± 2.32	10.52 ± 1.54	49.22 ± 3.79
Haddock	Raw	20.69 ± 4.38	10.01 ± 0.98	7.93 ± 0.88
	Fried	84.92 ± 12.12	8.39 ± 0.45	70.92 ± 8.12
Rainbow trout	Raw	56.78 ± 8.90	27.85 ± 3.98	22.01 ± 1.32
	Fried	92.95 ± 7.05	17.93 ± 1.02	78.03 ± 6.92

3.2. Platelet aggregating properties of lipid fractions

The extracted lipid fractions (TL, NL, PL) were tested for their ability to induce washed rabbit platelet aggregation or inhibit the PAF-induced platelet aggregation. The biological activity of each lipid fraction from all fish species, either raw or fried, along with their corresponding EC_{50} or IC_{50} value are shown in Table 2.

The lipid fractions of one group of fish species (golden trout, rainbow trout and sea bass) showed similar aggregatory patterns. Specifically, all TL fractions from either raw or fried samples exhibited strong aggregatory activities, which are mainly attributed to the polar lipids since PL fractions showed significantly higher aggregatory activities than the respective NL fractions from the same fish samples. All lipid fractions from raw golden trout and rainbow trout were slightly better aggregators than the respective lipid fractions from the fried samples. The lipid fractions from raw and fried sea bass showed similar aggregatory activities. The TL, PL and NL of sea bass, either raw or fried, were the strongest aggregating agents among the lipid fractions of these three fish species.

Table 2
Biological activity (aggregation of washed rabbit platelets or inhibition of PAF-induced platelet aggregation) of total lipids (TL), polar lipid (PL) and neutral lipid (NL) fractions of raw and fried fish species

Fish species	Processing	Lipid fraction	Action	EC_{50} (μg)[a]	IC_{50} (μg)[b]
Golden trout	Raw	TL	Aggregation	2.03 ± 0.672	
		PL	Aggregation	0.282 ± 0.101	
		NL	Aggregation	18.1 ± 2.4	
	Fried	TL	Aggregation	5.29 ± 1.22	
		PL	Aggregation	0.456 ± 0.134	
		NL	Aggregation	119 ± 23.2	
Coley	Raw	TL	Inhibition		122 ± 32
		PL	Inhibition		84.5 ± 7.89
		NL	Inhibition		1.60 ± 0.459
	Fried	TL	Aggregation	152 ± 34.2	
		PL	Inhibition		152 ± 38
		NL	–	180 ± 35.3	
Sea bass	Raw	TL	Aggregation	0.119 ± 0.054	
		PL	Aggregation	0.557 ± 0.139	
		NL	Aggregation	8.08 ± 1.06	
	Fried	TL	Aggregation	0.169 ± 0.098	
		PL	Aggregation	0.122 ± 0.061	
		NL	Aggregation	10.9 ± 2.85	
Plaice	Raw	TL	Aggregation/inhibition	0.298 ± 0.120	7.93 ± 1.02
		PL	Aggregation	0.295 ± 0.134	
		NL	Inhibition		33.3 ± 2.57
	Fried	TL	Aggregation/inhibition	2.15 ± 0.753	18.2 ± 3.21
		PL	Aggregation	2.03 ± 0.867	
		NL	Inhibition		148 ± 19.8
Haddock	Raw	TL	Inhibition		200 ± 43.2
		PL	Inhibition		40.1 ± 5.32
		NL	Inhibition		18.9 ± 1.95
	Fried	TL	Inhibition/aggregation	286 ± 43	0.0172 ± 0.0098
		PL	Inhibition		154 ± 52
		NL	Aggregation	216 ± 28	
Rainbow trout	Raw	TL	Aggregation	1.29 ± 0.233	
		PL	Aggregation	0.722 ± 0.194	
		NL	Aggregation	76.9 ± 8.28	
	Fried	TL	Aggregation	0.654 ± 0.117	
		PL	Aggregation	1.04 ± 0.143	
		NL	Aggregation	106 ± 21	

Values are reported as mean ± standard deviation of three independent replicates; each replicate is a pool of samples from three different specimen.
[a] The aggregatory activity of the sample was expressed as the amount of the sample in μg that is able to induce 50% of the maximum reversible aggregation of the respective sample.
[b] Values are expressed in μg of the lipid mixture that is able to induce 50% platelet inhibition against 2.5×10^{-11} M PAF.

A different pattern of biological action was found for plaice. The TL from raw and fried samples had a bimodal effect on platelets, aggregating them in low concentration and inhibiting their PAF-induced aggregation in higher concentrations. PL fractions were responsible for the aggregating properties of TL and NL fractions were responsible for the inhibitory actions of TL since they strongly aggregated platelets or inhibited PAF-induced aggregation, respectively, in all concentrations tested. The PL from raw plaice were better aggregators than the respective fractions from fried samples and the NL from raw plaice were better inhibitors than the NL from fried samples.

In haddock, all fractions obtained from the raw samples had inhibitory properties, while frying changed the biological actions of the NL fraction, which no longer inhibited, but aggregated platelets. Moreover, the TL fraction of fried samples showed a biphasic mode of action inhibiting platelet in low concentrations and aggregating them in higher ones.

Finally, in coley all lipid fractions from raw samples inhibited platelets, while in fried samples TL and NL aggregated platelets, and PL inhibited PAF-induced aggregation in all concentrations tested.

It could also be observed that distinctive different patterns were found in between the three fish-farmed species (golden trout, rainbow trout and sea bass) and the three open-sea fish species (coley, plaice and haddock). The farmed species had higher amounts of all classes of lipids than the corresponding amounts in the open

sea species (Table 1). It was also found that the farmed species exhibited strong aggregatory activities, whereas the open sea species showed mainly inhibitory biological activities (Table 2).

The existence of PAF antagonists in various foodstuffs is of major importance for their nutritional value considering the importance of platelet activation and thrombosis on cardiovascular diseases as well as the pivotal role of PAF in atherogenesis. Moreover, protective intervention studies against atherogenesis have shown that only specific PAF inhibitors (Feliste, Perret, Braquet, & Chap, 1989), olive oil polar lipids with PAF antagonists (Karantonis et al., 2004) and statins (Wierzbicki, Poston, & Ferro, 2003) are able to reduce atherogenesis in vivo. $\omega - 3$ Fatty acids, even though they inhibit solely events in atherogenesis since they suppress production of cytokines, modulate adhesion molecule expression, suppress rolling and monocyte adhesion to activated human endothelial cells, and they are not able to inhibit the whole phenomenon of atherogenesis when experimental animals are fed with atherogenic diet. $\omega - 3$ Fatty acids are recommended as a component in secondary prevention (Mayer et al., 2002). The consumption of PAF antagonists obtained from the diet is superior since they are of natural origin and therefore they imply no risks for human health as the toxic statins do. Fish is one of the main components of the Mediterranean dietary traditions and the beneficial effects of the Mediterranean diet could be explained in part by the presence of PAF antagonists in its foods. The presence of fractions with platelet activating properties, such as those found in rainbow trout, golden trout and sea bass, does not necessarily means that these fractions have prothrombotic effects since they are five to six orders of magnitude less active than PAF. The presence of such relatively inactive molecules in the bloodstream and their binding to PAF receptors could minimize the biological actions of PAF or in other words they could be considered as PAF inhibitors.

It should be mentioned that all these lipid fractions are a mixture of lipid molecules that can potentially have aggregatory or inhibitory properties. The final activity observed depends on both the relative ability of each molecule to aggregate platelets or inhibit the PAF-induced platelet aggregation and on the relative amount of each molecule in the mixture. In this respect, perspective a fraction that aggregates platelet may also contain inhibitory lipid molecules and the opposite. From a biochemical point of view, the structural elucidation and the quantitative determination of these lipid molecules is both interesting and challenging. However, from a nutritional point of view what is more important is the overall biological activity of the consumed mixture of biomolecules.

Potential sources of biologically active lipid molecules are the fish flesh in raw samples along with the frying medium in fried samples. Previous studies from our research team have found biologically active lipid fractions, obtained after HPLC separation, in raw cod[22]. Those fractions had both PAF-like and anti-PAF activities. We had also isolated and identified biologically active gangliosides from fish mackerel (Rementzis et al., 1997). Gangliosides are extracted to the PL fraction so some of the potent aggregating properties of PL fractions may also be due to this kind of polar lipids. Apart from gangliosides, a number of biologically active polar and neutral lipids were identified in mackerel, too. Those lipids possessed both PAF-like and anti-PAF activity and the structures of them were partially characterized (Rementzis et al., 1996). Moreover, other research groups identified the presence of PAF in rainbow trout tissues (Turner & Lumb, 1989) and catfish (Summers, al-Hassan, Thomson, Chun, & Criddle, 1991). Considering the fact that PAF is one of the most potent aggregating agents of platelets we can assume that even small amounts of it in the TL and PL fractions could lead to their strong aggregating properties. On the other hand, the molecular structure of the neutral lipids that were found in raw samples of golden trout, rainbow trout, sea bass and are able to induce platelet aggregation remains unclear and needs further studies.

The biological activity of the lipid fractions extracted from the frying samples is affected by both the absorption of the lipids of the frying medium in the fish flesh, the migration of substances from the fish flesh to the frying medium and from the thermal processing of the sample (Aro et al., 2000; Candela et al., 1997). Previous studies of our group had shown that TL, PL and NL from sunflower oil, which is used as the frying medium in this study, had inhibitory activities against PAF-induced aggregation (Karantonis et al., 2002). The lower aggregatory activity of TL, NL and PL from fried golden trout, rainbow trout and sea bass may be due to the absorption of substances with anti-PAF activities from the sunflower oil. Reduction of the amount of the aggregating lipids because of their extraction to the frying medium could also be another reason for the reduced aggregatory activity of the frying samples.

Heating of the samples during frying can lead to alterations of the molecular structure of lipids and thus to alterations of their biological activities. Oxidation and fragmentation are the main chemical changes that take place during heating and several oxidized and fragmented lipid products, such as oxidized phospholipids and cholesterol oxidized products, have been identified in previous studies (Addis, Park, Guardiola, & Codony, 1996; Tanaka, Tokumura, & Tsukatani, 1995). Oxidized phospholipids can have potent aggregatory, PAF-like properties (Tokumura, Sumida, Toujima, Kogure, & Fukuzawa, 2000). However, it seems that this is not the case in our study since, apart from haddock and coley, no increase of the aggregatory actions of lipid fractions were

found after heating of the samples. Probably, the relatively mild conditions of frying could not induce the formation of such oxidized lipids in our samples. The aggregatory properties of TL found in haddock and coley after frying were attributed to the NL fraction.

4. Conclusions

The lipid fractions of six widely consumed fish species possess strong biological activities in vitro in washed rabbit platelets, either acting as PAF antagonists with PAF-like activity or PAF inhibitors. Their mode of action depends on the fish species. To the best of our knowledge, this is the first study showing different biological activities of the farmed species as opposed to the open sea ones; the farmed species exhibited strong PAF-like activity whereas the open sea species showed mainly anti-PAF-like activity. The process of frying can alter their bioactivity leading to fractions of reduced aggregating activity. These changes are mainly attributed to the absorption of frying medium along with its bioactive substances in the fish (Panayiotou et al., 2000). The physiological importance of those alterations is dependent on bioavailability of the bioactive molecules in humans and their in vivo actions. However, considering the importance of platelet activation and thrombosis on cardiovascular diseases as well as the role of PAF in atherogenesis, the aforementioned properties of the fish lipid fractions could be a valuable index of their nutritional value.

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