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ORIGINAL PAPER

Circulating Salicylic Acid and Metabolic and Inflammatory Responses after Fruit Ingestion

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Abstract We hypothesized that fruit ingestion provides measurable amounts of salicylic acid (SA) and produces different metabolic and inflammatory responses compared to mere fruit sugars. In a randomized-crossover study, 26 healthy subjects received a peach shake meal (PSM) (SA: 0,06±0,001 mg/100 g) and a mixed sugar meal (MSM), consisting in an aqueous solution with the same sugars found in the peach shake. In order to control for the SA contribution from meals in the previous day, 16 subjects (Group 1) abstained from fruits and vegetables consumption the evening before trials, and 10 subjects (Group 2) maintained their usual diet. Circulating SA, glucose, insulin, free fatty acids, and interleukin-6 were determined. Basal SA was lower in Group 1 than in Group 2 (0.09± 0.02 vs. 0.30±0.03 μmol/l, $p<0.001$), peaked at 90 min in both groups (0.18±0.01 vs. 0.38±0.02 μmol/l, $p<0.01$) and remained above baseline ($p<0.05$) up to 3 h. Glycemia increased less after PSM at 15 min ($p<0.01$) with a lower average glucose excursion ($p<0.05$). Insulin peaked at 45 min with both meals but decreased less rapidly with PSM. Free fatty acids decreased more ($p<0.01$), and interleukin-6 increased less ($p<0.05$) with PSM. Dietary fruit intake increases the concentration of SA *in vivo*, and provides non-nutrients capable to modulate the inflammatory and metabolic responses to carbohydrates.

Keywords Fruit ingestion · Glucose · Inflammation · Insulin · Salicylic acid

Abbreviations

ASA	Acetilsalicylic acid
CRP	C reactive protein
FFA	Free fatty acids
FV	Fruit and vegetables
IL-6	Interleukin 6
MSM	Mixed sugar meal
PSM	Peach shake meal
SA	Salicylic acid
SE	Standard error

Introduction

Food is a complex mixture of macro and micronutrients producing a complex array of responses in humans including metabolic and inflammatory effects relevant to health status. Fruit and vegetables provide measurable amounts of phytochemicals that are often secondary metabolites of plants, where they exert hormonal and defensive activities. Several of them, like antioxidant or anti-inflammatory compounds, show healthy properties when introduced in humans [1].

Many bioactive compounds, recognized as protective agents for human health, nowadays have become real drugs, used to prevent and to treat several clinical conditions.

Salicylic acid (SA) is a striking example. SA plays a key role in the resistance to pathogens infection in plant [2, 3]. In

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the past, willow bark extract, rich in SA, was also used to soothe pain and inflammation. Since the last century, SA has been used in the synthesis of acetylsalicylic acid (ASA), a pro-drug prescribed for its analgesic and antinflammatory effect and, more recently, for the prevention and treatment of cardiovascular disease [4]. *In vivo*, ASA is rapidly deacetylated to SA, which prolongs its antinflammatory effect by a reversible inhibition of cyclooxygenase -1 enzyme and by inhibition of cyclooxygenase -2 gene transcription [5]. A regular ASA consumption at low-dose is associated with a reduced risk of cardiovascular disease and colon cancer [6].

SA is normally present in blood even in people not taking aspirin or other salicylates drugs [7]. Due to its presence in plants, it has been assumed that fruit and vegetables are the main sources of SA in humans. SA is ubiquitously present in fruits and vegetables, and herbs and spices contain the highest concentrations. However, published data are conflicting because of the different analytical techniques used [8]. According to Swain et al. [9] a western diet provides 10–200 mg/day of total salicylates. Other authors argue that SA contribution is 10–100 times lower [10].

Janssen et al. [11] correlated salicylates urinary excretion with the intake of dietary fibre and vegetable proteins in 17 healthy subjects following different diets. In vegetarian subjects, plasma levels of SA have been found to be higher than non-vegetarians, and to overlap with those of people taking 75 mg/day of ASA [7]. Urinary excretion of salicyluric acid, metabolite of phase I reactions from SA, was higher in vegetarian subjects than in non-vegetarians. However, SA urinary excretion was not different among free-ASA subjects and those taking 75 mg/day and 150 mg/day of ASA, indicating that SA is excreted mainly as salicyluric acid [12].

In a previous study, we demonstrated that a non-vegetarian diet providing 3–5 servings of fruit and vegetables per day, can ensure a substantial contribution of SA to the human body, and that circulating SA concentration is related to fruit and vegetables intake [13].

However, few studies evaluated the plasma concentrations of SA and the inflammatory parameters after a fruit meal consumption [14, 15]. The aim of this study was to investigate the effect of a fruit meal on SA plasma kinetic, and the metabolic and inflammatory responses in healthy subjects compared to an aqueous solution with the same sugars found in fruit.

Materials and Methods

Analysis of Fruit

Representative sample of *Prunus persica laevis,* variety Big Top, was analyzed to determine SA content and proximal composition. SA was determined by liquid-liquid extraction [10] by isotope dilution GC-MS, using deuterated internal standard d4-SA [16]. Proximal composition analysis (ash, moisture, soluble and insoluble fibre, protein) was carried out according to the AOAC methods [17, 18]. Soluble sugars, glucose, fructose and sucrose were performed by HPLC-amperometric detection.

Subjects and Experimental Design

Twenty-six volunteers (13 male and 13 female, Body Mass Index 23.36±0.59 kg/m^2, age 23.25±0.50 years) were recruited by advertisements among students of the University of Milan.

They underwent a preliminary medical examination to assess their health and nutritional status. Current drug therapies, dietary supplements, smoking status, chronic-degenerative diseases, acute inflammatory diseases, and metabolic disorders were exclusion criteria. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. Approval was obtained by the Institutional Ethical Committee and an informed consent was signed by all subjects. Since SA has a half-life that exceeds 6 h, 16 volunteers (Group 1: 8 male, 8 female) abstained from fruit and vegetable consumption from 6:00 pm of the day before the experiments, in order to better evaluate the net contribution that a fruit meal may provide on circulating levels of SA; the remaining 10 volunteers (Group 2: 5 male, 5 female) maintained their usual diet.

In a randomized, crossover study, with a wash-out period of one week, fasting subjects received a fresh home-made peach shake meal (PSM) and a mixed sugar meal (MSM), consisting in an aqueous solution with the same sugars found in fruit. The amounts of PSM and MSM were calculated in order to provide 0.71 g of total sugars (Σ glucose, fructose and sucrose) per kilogram of body weight. The percentage composition of sugars (fructose, glucose and sucrose) and the final weight of the meals were identical.

The protocol started at 08:00 am after an overnight fast from 10:00 p.m. Venous blood samples were obtained from a cannula needle before meal consumption and after 15, 30, 45, 60, 90, 120, and 180 min. During this time, subjects were allowed only to drink no sparkling water.

Blood aliquots were stored at −80 °C until examination.

Blood Assays

Circulating SA was determined by gas chromatography–mass spectrometry analysis, using a method previously published by us and suitable to detect SA in concentrations found in aspirin-free persons (detection limit of 0.6 ng and a quantification limit of 2 ng) [14].

Plasma glucose was measured by oxidation with YSI 2300 STAT Plus™ Glucose & Lactate Analyzer. Insulin was determined by ELISA kit (DiaMetra S.r.l, Segrate, Italy). Triglycerides were determined by enzymatic kit (Sentinel CH. SpA, Milan, Italy). The measurement of free fatty acids was carried out by enzymatic-colorimentric reaction (Roche Diagnostics, Mila, Italy). Commercial immunoassays kits were used to measure Interleukine-6 (IL-6) and C-reactive protein (R&D Systems, Inc., France).

Statistical Analysis

All results were reported as mean ± standard error (SE). Outcomes were analyzed using repeated measures *t*-test. Statistical significance was based on 95% limits (α=0.05), where appropriate data were log-normalized prior to statistical analysis, as reported in the text. Statistical analysis was carried out using the program SPSS version 17.

Results

Table 1 shows macronutrient, total fibre, total polyphenols and SA content in Big Top peaches.

No SA was detected in the MSM solution.

The time course of plasma SA concentration is shown in Fig. 1.

Basal SA concentration was lower (p<0.001) in Group 1 (0.09±0.02 μmol/l) than in Group 2 (0.30±0.03 μmol/l). After fruit ingestion SA levels in Group 1 increased significantly after 30 and 60 min (p<0.001) and peaked at 90 min (p<0.01) at 0.18±0.015 μmol/l. Two hours after meal ingestion SA concentration tended to reduce but remained above baseline (0.14±0.01 μmol/l, p<0.05) also after 3 h. In Group 2, after peach shake ingestion, SA time course was similar to the Group 1, and reached the maximum concentration (0.38±0.02 μmol/l, p<0.05) at 90 min, and decreased thereafter remaining above baseline (0.35±0.03 μmol/l, p<0.01) at 3 h.

Table 1 Macronutrient, total fibre, total polyphenols and SA content in Big Top peaches

Moisture (%)	85.91±0.001
Tot sugars (g/100 g)	11.44±0.10
Glucose (g/100 g)	1.26±0.01
Fructose (g/100 g)	1.30±0.03
Sucrose (g/100 g)	8.92±0.03
Protein (g/100 g)	0.065±0.005
Ash (g/100 g)	0.30±0.002
Fibre (g/100 g)	0.6±0.03
Salicylic acid (mg/100 g)	0.06±0.001
Polyphenols (mg/100 g)	89.86±4.36

Data are expressed as means and SE (n=4)

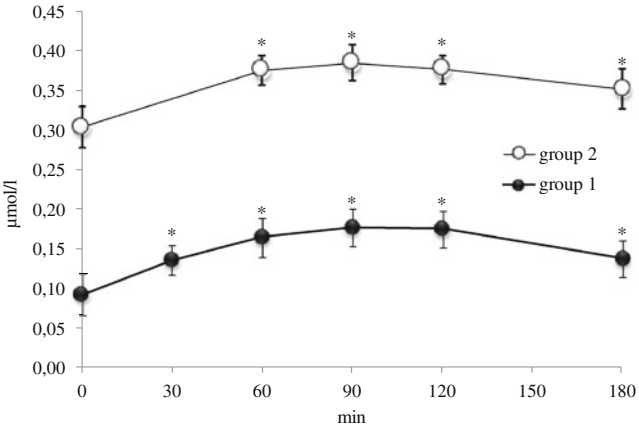

Fig. 1 Plasma time-course of SA after a peach shake meal (PSM) in subjects who abstained from fruit and vegetable consumption on the evening before the test (Group 1, n=16), and in subjects with a free diet (Group 2, n=10). Each value is presented as mean ± SE. *, p<0.05 vs. t0

Within each group, the concentrations of SA prior to the ingestion of MSM and PSM were not different. After MSM, circulating SA levels did not change from basal.

Since we administrated the same amount of sugars to each subject and we could not find any effect of the peach type on the metabolic responses analyzed, we decided to report the results of the two groups pooled together. In Table 2 the concentrations of plasma glucose, insulin, FFA, CRP and IL-6 after PSM and MSM ingestion are reported.

Plasma glucose concentration increased more slowly (p< 0.01) in PSM with respect to MSM (+9.69±0.70 vs. +28.21± 3.15 mg/dl at 15 min), and decreased less in the last hour (p< 0.001) determining a smaller average glucose excursion than MSM (39.15 mg/dl vs. 50.42 mg/dl, p<0.05). After meals ingestion insulin peaked at 45 min with both PSM (41.49± 5.94 μU/ml) and MSM (38.40±5.56 μU/ml). In the last 2 h insulinemia decreased less rapidly with PSM than MSM. Free fatty acids were percentually more suppressed after PSM ingestion with respect to MSM (−72.04% vs. −53.31%, p< 0.01) in the first 2 h, and lower than baseline even after 3 h from PSM consumption (p<0.05).

We did not find significant differences between the meals in C-reactive protein (CRP) either basally and after the meals consumption. In contrast, after the first hour IL-6 concentration tended to increase both with PSM and MSM, but less with PSM (2.48±0.89 pg/ml than 9.38±2.47 pg/ml, p<0.05) (Fig. 2).

Discussion

Our results confirm that salicylic acid is normally present in the blood of people who do not take salicylates drugs. Fruit

Table 2 Metabolic and inflammatory markers

Time (min)	Plasma glucose (mg/dl)		Insulin (μU/ml)		FFA (μmol/l)		CRP (ng/ml)		IL6 (pg/ml)		
	PSM	MSM	PSM	MSM	PSM	MSM	PSM	MSM	PSM	MSM	
0	86,28±1,2	84,28±1,1	9,85±1,45 α	7,27±0,92 α	0,52±0,07 α	0,35±0,05 α	1086±402	1295±475	1,98	0,44	1,97	0,25
15	95,13±1,63 * α	114,1±4,15 * α	30,17±4,16 *	34,57±3,72 *	ne	ne	ne	ne				
30	112,9±2,25 *	111,5±3,32 *	37,07±4,08 *	32,72±5,13 *	0,34±0,04 * α	0,2±0,02 * α	ne	ne	1,18	0,15	1,12	0,16
45	111,7±3,93 *	113,2±10,3 *	41,49±5,94 *	38,4±5,56 *	ne	ne	ne	ne				
60	89,00±2,71	84,36±5,72	27,17±4,28 * α	18,89±2,98 * α	0,12±0,01 *	0,15±0,01 *	ne	ne	2,83	0,42	3,12	0,54
90	84,81±1,67	77,95±4,53	19,01±3,09 * α	12,21±3,62 α	ne	ne	976±364	662±323				
120	84,58±1,67 α	79,2±2,26 * α	14,1±2,27	11,56±2,33	0,1±0,01 * α	0,27±0,05 α	ne	ne	3,27	1,36	4,84	2,42 *
180	81,64±1,22 * α	77,16±1,35 * α	7,71±1,02 α	5,62±0,92 α	0,32±0,05 * α	0,49±0,05 * α	1084±360	1156±452	2,48	0,89 *	9,38	2,47 *

Data are expressed as mean ± SE (n=26)

n.e: not evaluated

α: $p<0.05$ PSM vs. MSM

*:$p<0.05$ vs. t0

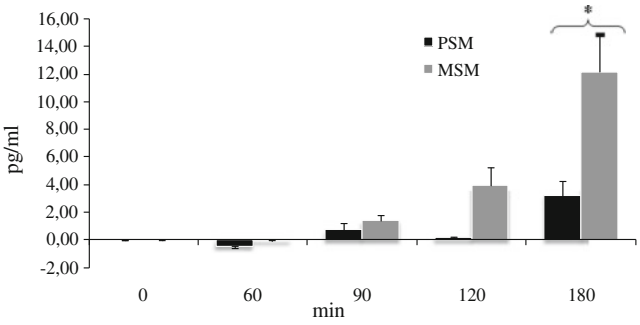

Fig. 2 Absolute change of the IL-6 after a peach shake meal (PSM) and a mix sugar meal (MSM) in 26 healthy subjects. Each value is presented as mean ± SE. *, $p<0.05$ PSM vs. MSM

and vegetables are the main dietary sources of salicylic acid, and subjects on a free diet showed plasma concentrations of salicylic acid three times higher than individuals who abstained from fruit and vegetables consumption on the previous evening. Nonetheless, in the latter individuals, circulating salicylic acid was still measurable. The reason could be found in its long half-life [19], but the existence of endogenous salicylic acid synthetic pathways, as recently suggested by Paterson [20], could be an alternative explanation.

In our study we demonstrated that a peach meal, equivalent to two servings of fruit, can provide an appreciable amount of salicylic acid. Salicylic acid concentration tended to double in the first hour and remained elevated after 3 h after fruit consumption.

An interesting point of discussion is whether the low dietary concentrations of salicylic acid in humans have a nutritional and biological role. Even though acetylsalicylic acid is clinically used at doses that are two orders of magnitude greater than the doses naturally delivered in our study, we demonstrated that after a fruit meal, basal circulating salicylic acid may be reaching concentrations comparable with those found by Blacklock et al. [7] in vegetarians and may overlap those of subjects chronically taking 75 mg/day aspirin. Moreover it is interesting to notice that the concentrations of salicylic acid achieved in our study are similar to those found by Xu et al. [21] to be effective in inhibiting cyclooxygenase-2 gene transcription in human umbilical vein endothelial cells. These remarks therefore suggest that circulating salicylic acid levels found in our study after fruit intake could play "*in vivo*" a biological role in terms of anti-inflammatory effect.

The meal is considered a pro-inflammatory stimulus [22, 23] and recent studies reported evidence for a pro-inflammatory effect of specific nutrients consumption and of food matrixes [24], producing an increment in Nuclear Factor-kappa B translocation in the nucleus and in the expression of inflammatory cytokines. In our study, we investigated the metabolic and inflammatory responses of a carbohydrate meal administered in two contexts either as a complex fruit matrix or as a watery solution. The watery

solution determined a greater glycemic excursion with a trend to a reactive hypoglycemia that led to a greater insulin suppression and to a rebounded free fatty acids increment in the third hours. Concerning the inflammatory response, even thought we did not find any difference in C-reactive protein levels, we found that if equivalent amounts of carbohydrates are consumed as a peach, interleukin-6 increment is lower. Therefore, our results proved a lesser proinflammatory action of fruit. Although our findings suggest that this effect may be due to salicylic acid derived from peach meal ingestion, we cannot exclude the contribution of other phenolic compounds, such as caffeic, ferulic, sinapic, *p*-coumaric, cinnamic and gallic acids, and flavonoids that are widely present in fruit and vegetables and have well-known anti-inflammatory properties [25, 26].

In conclusion, the present study confirmed that fruit and vegetables affects salicylic acid serum levels in humans and shows that fruit ingestion has beneficial effects on postprandial metabolic and inflammatory responses. However, the mechanism of the possible anti-inflammatory effect of salicylic acid in relation to other bioactive compounds derived from fruit ingestion remains to be elucidated. Finally, in order to confirm the possible use of salicylic acid as a biomarker of fruit and vegetable consumption, it will be useful to carry out studies on the content of salicylic acid and other phenolic compounds in foods, their bioavailability in man and the consequent effects on human health.

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