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The hypotriglyceridemic effect of sciadonic acid is mediated by the inhibition of $\Delta 9$ -desaturase expression and activity

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Abbreviations

Scia: sciadonic acid; SCD1: Stearoyl-CoA Desaturase 1; FADS: Fatty Acid Desaturase; ECM:

ExtraCellular Matrix; FBS: Fetal Bovine Serum; SREBP1c: Sterol Regulatory Element-Binding Protein

1c; ACC: Acetyl-CoA Carboxylase; FAS: Fatty Acid Synthase; Elovl6: Fatty Acid Elongase 6; MTTP:

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Microsomal Triglyceride Transfer Protein; ApoB: Apolipoprotein B; PPAR α : Peroxisome Proliferator Activated Receptor α ; FAT/CD36: Fatty Acid Translocase/CD36; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; TLC: Thin Layer Chromatography, VLDL: Very Low Density Lipoprotein

Keywords

Human, Rat, Sciadonic acid, Stearoyl-CoA Desaturase 1, Triglycerides

Abstract

Scope

Sciadonic acid (Scia; 20:3 Δ 5,11,14) is a distinctive fatty acid (FA) with a polymethylene-interrupted double bond at C5. It is specifically found in seeds from gymnosperms such as pine nuts. Published papers describe a decrease in liver and plasma triacylglycerols in rats fed with this nutriment. The present study sought to identify the action mechanism of Scia on triacylglycerol synthesis. In this way, its nutritional effect on FA metabolism involving the Stearoyl-CoA Desaturase 1 (SCD1) was investigated.

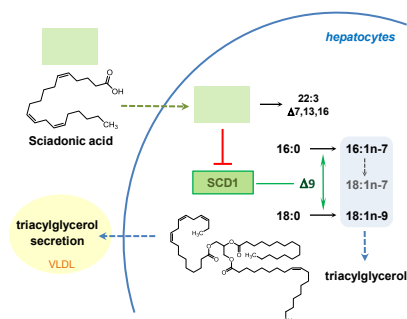
Methods and results

Scia was discerned in trace amount in various tissues of rats and in human serum. It was produced by Δ 5-desaturation of 20:2n-6 in human transfected SH-SY5Y cell lines and also in rat hepatocytes. When Scia was incubated with cultured hepatocytes as a nutrient, the cellular FA profile was modified. In particular, the proportion of the monoenes (18:1n-9, 18:1n-7, 16:1n-7) were all decreased, correlating to the reduction of triacylglycerol amounts. This effect was mediated by the inhibition of SCD1 expression. Furthermore, Scia, as well as 20:3n-6 and 20:3n-9 but not 20:3n-3, strongly inhibited the SCD1 activity measured on liver microsomes.

Conclusion

Overall this study showed that Scia, despite its unusual structure, contributes to the FA metabolism and reduced triacylglycerol release by inhibiting SCD1 activity.

Sciadonic acid is a distinctive plant fatty acid (FA), which was identified in rat tissues and human serum. When supplied to cultured hepatocytes, it reduced triacylglycerol synthesis through inhibition of SCD1. This enzyme Δ^9 -desaturates saturated FA, producing monoenes such as 16:1n-7 and 18:1n-9. Despite its unusual structure, sciadonic acid contributes to the FA metabolism like a classical methylene-interrupted polyunsaturated FA.



1. Introduction

Sciadonic acid (Scia) belongs to the family of $\Delta 5$ -olefinic acids specifically abundant in gymnosperms and is consumed through edible seeds such as pine nuts. This particular fatty acid (FA) family comprises dienes, trienes or tetraenes all with *cis* saturations. Such FA have either 18 or 20 carbons, and display a non-malonic structure at the carboxyl end such as $\Delta 5,9$ or $\Delta 5,11$ (**Supplemental Figure 1A**). Indeed they display a polymethylene-interrupted double bound at C5, which corresponds to the closest unsaturation point in the molecule from the carboxyl end for FA found in plants. However, an ethylenic bond at C3 may be observed in FA in the plant families *Compositae* and *Bignoniaceae*, but these are with a *trans* configuration (18:1 $\Delta 3t$, 18:3 $\Delta 3t,9c,12c$, 18:4 $\Delta 3t,9c,12c,15c$) [1]. $\Delta 5$ -olefinic acids are usually given a biological name in reference to the most abundant source. Seven FA are arise from gymnosperms and their profile represents a good marker of phylogeny, in parallel to botanic observations and genetic characterizations. Six of these FA are common to almost all conifers, whereas ephedrenic acid is only found in *Ephedraceae* and *Ginkgoaceae*.

Despite its unusual structure, Scia follows the pattern of the other 20:3 isomers found in small amounts in animal tissues, and it displays two of the three double bounds also present in 20:3n-9 ($\Delta 5,8,11$), 20:3n-6 ($\Delta 8,11,14$) and 20:3n-3 ($\Delta 11,14,17$; **Supplemental Figure 1B**). Specifically, Scia can be incorporated in animal cell membranes when included in the diet, by substituting, in part, arachidonic acid (20:4n-6), which is substantially esterified in phospholipids such as phosphatidylinositol [2, 3]. Consequently, different published works underlined the effect of Scia as modulator of the inflammation response by reducing the production of pro-inflammatory mediators synthesized from arachidonic acid, as the Scia molecule lacks the $\Delta 8$ [4–7]. In addition, Scia was

shown to modulate lipid metabolism in rats and particularly by reducing the triacylglycerol concentration in the plasma and liver of animals fed with a diet enriched with *Torreya nucifera* or *Biota orientalis* seed oil [8–11].

In the first part of this study, samples of rat tissues and human sera were screened for the presence or absence of Scia. In the second part, the effect of Scia as a hypotriglyceridemic modulator was investigated by studying the $\Delta 9$ -desaturase. This enzyme is also known as Stearoyl-CoA Desaturase 1 (SCD1) in hepatic cells and this responsible for the *de novo* synthesis of the oleic (18:1n-9) and palmitoleic (16:1n-7) acids, that are involved in the process of triacylglycerol formation. It was hypothesized that Scia may inhibit the expression or activity of SCD1, considering its distinctive structure that is close to the typical and functional long chain polyunsaturated FA.

2. Materials and methods

2.1 Human and animal tissue sampling

Blood from non-fasting human subjects (n=11) was collected and fractioned to quantify the Scia in serum samples. Subjects ranged from 20 to 41 years-old and comprised 36% men and 64% women (Laboratoire Le Minous, Bourg L'Evêque, Rennes, France). In the case of animal work, all protocols performed complied with the European Union Guideline for animal care and use (2010/63/CEE; decree 2013-118; authorization #01305.02). Male Sprague-Dawley rats (7-8 weeks-old) were purchased from the Janvier Labs Breeding Center (Le Genest-Saint-Isle, France). Animals had access to water and food *ad libitum* (aging diet, Special Diet Services, Augy, France). The litter was made from pine shaving that contained Scia (242 μ g/g). Non-fasted rats for liver isolation and fasted rats for liver perfusion were anesthetized with intraperitoneal injections of ketamine (100mg/kg,

Imalgene®1000, Merial, Lyon, France) and xylazine (10mg/kg, Rompun® 2%, Bayer Animal Health, Puteaux, France).

2.2 Cell culture

Rat primary hepatocytes were isolated by a collagenase perfusion using liberase™ (Sigma-Aldrich) as described in earlier work [12]. Four hours after plating in Primaria™ dishes (100mm diameter), cells were treated either with Scia (Lipidox, Stockholm, Sweden) or with 20:2n-6. They were cultured in DMEM with or without 7% fetal bovine serum (FBS) and some of each were also enriched with insulin (1µmol/L). Hepatocytes were then isolated using accutase® (Sigma-Aldrich) at indicated times of culture. In this study, the different conditions of different cultures (with or without FBS and insulin) were used to stimulate the expression of SCD1 and favor the release of triacylglycerols. SH-SY5Y human neuroblastoma cells (received from J.M. Alessandri, UR-INRA909, Département Alimentation Humaine, Jouy-en-Josas, France) were used as described elsewhere [13].

2.3 Cell treatment with fatty acids

Scia (100µmol/L) was supplied as an albumin complex. Briefly, saponification had been carried out with 0.5mol/L NaOH in methanol for 30min at 70°C. Scia was then dissolved in DMEM enriched with 1% bovine serum albumin free of any FA (Serva, Coger, Paris, France). This solution was adjusted to pH 9 and incubated over night at room temperature. The medium complexed with Scia was finally adjusted to pH 7.4 and passed through a 0.2µm filter. In experiments using 20:2n-6 (**Figure 2**), the FA was directly diluted to 60µmol/L in the culture medium using a 1% ethanol solution.

2.4 Lipid analyses

Lipids from human and rat tissues were extracted using Folch's method [14], whereas lipids from cells, from the extracellular matrix (ECM obtained from accutase[®]) and from the culture medium were all extracted following Hara's method [15] after acidification by 3mol/L HCl. Lipids were saponified with 0.5mol/L NaOH in methanol at 70°C for 20 min and methylated with BF₃ (14% in methanol) at 70°C for 15 min. FA methyl esters (FAME) were separated using a 7890N Agilent GC equipped with a BPX70 capillary column and coupled to a 5975C mass spectrometer (Agilent Technologies, Les Ulis, France) following procedures previously described [16]. Agilent MSD ChemStation software was used for data acquisition. Components were identified from the retention time of FAME standards and also by using the NIST mass spectral library (version 2.01). For the human sera, identification of Scia present was confirmed following separation of the FAME components on AgNO₃-impregnated silica Thin Layer Chromatography (TLC, cyberlipid.org). Results were given as the mass percentages of the total identified FA and concentrations were calculated by using 17:0 as internal standard. The results of the Scia metabolism are preferentially presented as the % of the total FA excluding Scia. In that case, percentages were recalculated without taking into account the GC area of Scia. Thusly, the incorporation of Scia was distinguished from its effect on FA metabolism. Noting that the concentration of Scia was negligible in tissues (less than 0.1%, **Figure 3A**), it was deduced that its incorporation (around 8% of total FA, **Figure 3A**) led to a reduction of the other FA proportions. The same calculation was done with 20:2n-6 in **Figure 2C**. In some experiments, triacylglycerols were quantified directly on the culture medium and on the cell extracts by using a BioMérieux kit (Bruz, France).

2.5 Triacylglycerol staining

Lipid droplets containing triacylglycerols were observed on hepatocytes cultured for 72h with and without Scia, in DMEM supplemented with 7% SVF and containing 1 μ mol/L of both insulin and dexamethasone. Lipids were stained with oil red O (CliniSciences, Nanterre, France) following a procedure described by the manufacturer. Cells were observed at a magnification of 40x by an Olympus E-450 camera coupled to an Olympus IX51 microscope (Olympus, Paris, France).

2.6 [3 H]Triacylglycerol synthesis and release

Hepatocytes were cultured in PrimariaTM dishes (60mm diameter) with DMEM supplemented with 7% SVF and containing 1 μ mol/L of both insulin and dexamethasone. Cells were treated with Scia for 48h. The medium was then removed and replaced by one without Scia. [3 H]-Glycerol (5 μ mol/L, 2 μ Ci/dish) was incubated for 18h. Lipids from the medium, from the hepatocytes and from the ECM were all fractionated by silica TLC using the mixture hexane: diethyl ether: acetic acid 80:20:1 (v/v) as the mobile phase. Spots of triacylglycerols were scrapped off and radioactivity was quantified by scintillation counting.

2.7 mRNA relative quantification

Total RNA was extracted using Trizol[®] (Life Technologies, Saint-Aubin, France) and reverse transcribed by using the high capacity cDNA RT kit (Applied, Fisher, Illkirch, France). Real-time PCR of *Scd1*, *Fads1*, *Fads2* and *Fads3* (Taqman[®]) was carried out as described in previous publications [13, 17]. For the other genes, experiments were performed by using SYBR[®]Green PCR (Bio-Rad, Marnes

La Coquette, France) with specific primers from Eurogentec (Angers, France, **Supplemental Table 1**). The mRNA expression was evaluated as a delta Cycle threshold ($\Delta Ct = Ct_{\text{gene}} - Ct_{18S}$ for Taqman[®] and $\Delta Ct = Ct_{\text{gene}} - Ct_{\text{GAPDH}}$ for SYBR[®]Green) with a 100% efficacy verified by using a standard curve. Results obtained from triplicates per cell culture were presented from the $2^{-\Delta Ct}$ calculation as explained in the figure captions.

2.8 $\Delta 9$ -desaturase expression

The expression of $\Delta 9$ -desaturase was estimated by western-blot after SDS-PAGE on 15 μ g of cell extract proteins. Goat anti-SCD1 (Santa-Cruz Biotechnology, Heidelberg, France) and rabbit anti- β actin (Sigma-Aldrich) were used following the manufacturer instructions. Primary antibodies were coupled to horseradish peroxidase-conjugated anti-IgG and the peroxidase activity was determined by chemiluminescent detection using Immobilon reagents (Millipore, Molsheim, France). The apparent molecular mass of proteins was determined by the use of a standard curve constructed with the Kaleidoscope marker (Bio-Rad) in 10% SDS-PAGE. The density of bands was estimated by using Image J software (US National Institute of Health, www.nih.gov).

2.9 Assay for $\Delta 9$ -desaturase activity

Liver was fractioned in phosphate buffer solution containing 0.25M sucrose to recover the post-mitochondrial supernatant as described in previous work [17]. This post-mitochondrial supernatant was treated with Scia (15 μ g/mg of proteins) or with ethanol as vehicle, then keeping for 10min in ice prior to the activity measurement. Isomers of 20:3 (n-9, n-6, n-3) were tested in parallel as the control. The enzymatic activity was then assayed in duplicate with [1-¹⁴C]-16:0 and [1-¹⁴C]-18:0

substrates (60 μ mol/L, 10mCi/mmol). The FA were separated by HPLC and quantified by scintillation counting.

2.10 Statistics

Statistical analysis was performed using R software. For the human tissue samples, the variables were studied by principal component analysis. For the rat tissue samples, the effect of Scia was evaluated by the difference of mean rank values (Mann-Whitney and occasionally Kruskal-Wallis). In some experiments, a paired-t test was done as indicated in the figure legend. Correlations were evaluated by Pearson correlation coefficients and the analysis of variance was done by ANOVA. Significant differences (at least $p < 0.05$) were marked by an asterisk or occasionally by different letters.

3. Results

3.1 Sciadonic acid is present in human and rat tissue

Scia is known as an unusual plant FA that is widely found in gymnosperms. Nevertheless in this study, Scia was also shown to be naturally present in human and rat tissue (**Figure 1**). It represented around 0.1% of the total FA in human serum. The Scia peak was almost imperceptible in the chromatograms but the identification of Scia was still achieved on samples of serum from eleven individuals by the specific elution (**Figure 1A**) coupled with mass spectrum. Separation on silver nitrate-TLC confirmed the presence of Scia as well as the other 20:3 isomers present in human serum. The elution time of Scia corresponded to the purified standard (from Lipidox) used in this

trial. The same result was obtained by using pine oil purified by Polaris and which contained more than 30% of Scia.

Sampling from the human subjects showed otherwise a wide range of lipid profiles (**Table 1**). A significant and high correlation between triacylglycerols and monoenes was observed ($p=0.00041$), especially for SCD1 products such as 18:1n-9 ($p=0.00036$; not shown). On the contrary, serum triacylglycerols were inversely correlated with polyunsaturated FA ($p=0.0021$). Trends were only observed between Scia and triacylglycerols ($p=0.1073$) or 18:1n-9 ($p=0.0993$). Surprisingly Scia was negatively correlated to human age ($p=0.0031$, **Figure 1B**), but this correlation was the only one found for these two variables. These results thus underline the decrease of Scia concentration in serum with the increasing age of individuals. The random selection of subjects was however still dominated by relatively young people. In the study using rats, Scia in tissues was usually found at around 0.1 - 0.2%, and up to 0.4% in plasma (**Figure 1C**). In rat liver, the Scia content reached 50ng per mg of tissue. These results suggest the ubiquitous presence of Scia in the organism, although only in trace amounts.

3.2 Sciadonic acid is synthesized from 20:2n-6 by $\Delta 5$ -desaturase

As Scia was shown to be naturally present in rat tissues, its synthesis may be expected with mammalian desaturases since no Scia was found in the rodent diet. To verify this hypothesis, we transfected SH-SY5Y cells with *Fads1*, *Fads2* or *Fads3* genes, coding for rat $\Delta 5$ -desaturase, $\Delta 6$ -desaturase and FADS3 respectively (**Figure 2A and 2B**). The results show that Scia was formed after $\Delta 5$ -desaturation of 20:2n-6, whereas the $\Delta 6$ -desaturase induced a $\Delta 8$ -desaturation producing 20:3n-6. FADS3 displayed no enzyme activity. When 20:2n-6 was incubated with primary hepatocytes (**Figure 2C**), Scia was also produced with the endogenous $\Delta 5$ -desaturase but only at a low level (0.48% in mean with or without FBS) as compared to transfected cell lines expressing the

recombinant $\Delta 5$ -desaturase (4.2% with *Fads1*, **Figure 2B**). Partial β -oxidation of 20:2n-6 was however favoured noting a substantial rise of 18:2n-6 (**Figure 2C**). By comparison, 20:3n-6 was unchanged with the incubation of 20:2n-6. In conclusion, hepatocytes preferentially metabolized 20:2n-6 by $\Delta 5$ -desaturation producing Scia, than by $\Delta 8$ -desaturation generating 20:3n-6. These cells naturally neosynthesized a FA with a polymethylene-interrupted double bond, instead of producing more typical methylene-interrupted structure.

3.3 Sciadonic acid is incorporated into rat hepatocytes

In the next step, Scia was incubated with primary hepatocytes for 24h (**Figure 3A**). The treatment with insulin was used to modulate the cellular entry and metabolism of FA. The subsequent results showed that the control cells naturally contained a small amount of Scia (0.08% and 0.04% for those without and with insulin respectively). The proportion of FA increased to 8% when Scia was supplied to the culture medium. Insulin increased the amount of Scia in the incubated cells (from 78.4 nmol/dish to 135.9 nmol/dish) without impacting its proportion (around 8% of total FA). Insulin favored the entrance of nutrients such as FA into cells. In this way, it also influenced the global repartition of Scia in the culture compartments (**Figure 3B**). Indeed the insulin increased the FA content in cells to the detriment of the medium and the ECM. In consequence, incubated Scia was more prominent in cells although it was recovered from the medium and the ECM as well after 24h of culture.

3.4 Sciadonic acid is metabolized in rat hepatocytes

Attention now moved to Scia metabolism in cells. After incubation of Scia for 24h, the FA profile in the hepatocytes was analyzed (**Figure 4**). Two specific metabolites were identified when Scia was

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supplied to the culture medium (**Figure 4A**). The first arose from the elongation of the Scia molecule to form 22:3 Δ 7,13,16 but only in a very small proportion. The second metabolite arose when Scia was possibly β -oxidized into 16:2n-6 (Δ 7,10) as suggested by Tanaka [18]. Consequently, 18:2n-6 proportion was increased following the elongation of 16:2n-6 derived from Scia. As a result, the subsequent elongated product 22:4n-6 was especially observed, whereas the proportion of arachidonic acid tended to decrease (not shown). Additionally, insulin did not significantly impact the FA proportions but rather increased the FA amounts. Collectively, these results underline the overall increase of n-6 FA in cells treated with Scia (**Figure 4B**). On the contrary, incubation with Scia reduced the proportions of monounsaturated FA, and in particular 18:1n-9 (**Figure 4B**). When 16:1n-7/16:0 and 18:1n-9/18:0 ratios were calculated, a slight effect of Scia was revealed. Since Scia lowered monoenes without greatly impacting the ratios, then *de novo* synthesis of FA (i.e. specifically the 16:0 and 18:0) may be affected as well. Thus the expression of enzymes involved in the FA metabolism in presence of Scia was investigated.

3.5 Sciadonic acid reduces the expression of desaturases and elongase from rat hepatocytes

Monoenes are synthesized from 16:0 and 18:0 through the action of SCD1, which introduces a *cis* double bond at C9 producing either 16:1n-7 (Δ 9), elongated then in 18:1n-7, or 18:1n-9 (Δ 9).

Considering the monoene proportions previously observed, the effect of Scia on SCD1 expression was investigated further. The results showed that Scia significantly reduced the protein level of SCD1 in hepatocytes cultured in different conditions (**Figure 5A**). When SCD1 was overexpressed from FBS and insulin stimulation, the effect of Scia was more pronounced and was in adequacy with gene expression estimated by qPCR (**Figure 5B**). By comparison with the other desaturases, a similar profile with *Fads1*, *Fads2* and *Fads3* was found. Scia depressed the gene expression as well but to a lower extent than for *Scd1*. In this way, Scia induced a reduction of monoenes by lowering SCD1

expression. This result may also be explained by considering preceding mechanisms. It was hypothesized that Scia may have modulated the neosynthesis of FA precursors. The results produced showed that *acc*, coding for the enzyme catalyzing the malonyl-CoA synthesis from acetyl-CoA, was not affected. Additionally, the expression of *fasn*, which is implied in the synthesis of 16:0 from malonyl-CoA, tended to be reduced. By contrast, Scia significantly inhibited the insulin-induced expression of *elovl6*, that is involved in the elongation of 16:0 into 18:0 (**Figure 5B**). However, no correlation between this gene expression and the saturated FA content of cells was observed as Scia did not significantly affect the level of 16:0 or 18:0 (not shown). In conclusion, Scia modulated the expression of specific enzymes implicated in the *de novo* synthesis of FA, and in particular of SCD1. Based on that observation, the consequences on the *de novo* formation and secretion of triacylglycerols were further analyzed.

3.6 Sciadonic acid reduces the triacylglycerol secretion in rat hepatocytes

When hepatocytes were cultured for 3 days in a medium containing FBS and insulin, Scia reduced monoenes in cells after 24h (Kruskal-Wallis $p=0.05$; **Figure 6A**). Scia also decreased the SCD1 expression that was particularly pronounced as a function of time and under FBS and insulin incubation (**Figure 6B**). Triacylglycerol amounts in the medium and in the cells increased with the culture time (**Figure 6C**). However, this increase was significantly reduced beyond 48h when cells were incubated with Scia. In addition, the triacylglycerol content was positively correlated with the concentration of 18:1n-9 (**Figure 6D**) as well as with 16:1n-7 and 18:1n-7 (data not shown). Nonetheless when cells were treated with Scia, triacylglycerols and 18:1n-9 were both reduced after 48h (**Figure 6D**). A comparable result was observed in cells stained with oil red O for 72h of culture (**Figure 6E**). Scia reduced the number of lipid droplets, without affecting their morphology. Since the triacylglycerol decrease in the medium may result from a reduction of cell synthesis or intake, and

noting the difference between cell and medium results, the triacylglycerol secretion by measuring the release of [³H]-glycerol (**Figure 6F**) was also checked. The results produced confirmed that Scia reduced neosynthesized [³H]-triacylglycerols by 27% in the experimental conditions of this study, and in all compartments of the cell culture.

3.7 Sciadonic acid modulates the activity of hepatic SCD1

Since Scia inhibited SCD1 expression resulting in the reduction of triacylglycerol neosynthesis, an *in situ* effect of Scia was thus hypothesized. The effect of Scia and 20:3 isomers was tested on SCD1 activity measured on liver samples. Scia and 20:3 isomers were incubated with post-mitochondrial supernatants prior to the enzyme assay. In the study conditions, 15µg/mL of Scia corresponded to 50µmol/L, which is close to the radiolabeled substrate concentration (60µmol/L). In this way, the results produced showed that Scia strongly reduced the SCD1 activity measured on both of the substrates (**Figure 7A**). The effect was similar on [¹⁴C]-16:0 and [¹⁴C]-18:0 as the inhibition being evaluated as around 50%. To consider factors other than the potential bias introduced with the non isocaloric control, different alternative FA were tested (**Figure 7B**). Thus when Scia was compared with the other 20:3 isomers, a differential effect depending on the unsaturation combinations was found. Indeed, 20:3n-3 did not impact on the [¹⁴C]-18:1Δ9 synthesis, whereas 20:3n-6 reduced the SCD1 activity by 69%, 20:3n-9 by 58% and Scia by 46%.

3.8 Sciadonic acid reduces the expression of genes implicated in lipogenesis

Long chain polyunsaturated FA that are usually found in mammals are known to modulate gene expression of the different actors involved in the secretion of triacylglycerols. The potential impact of Scia independently of its regulation on SCD1 was investigated. The first observation was that Scia

reduced by 62% the gene expression of sterol regulatory element binding protein (SREBP) 1c, implied in the stimulation of lipogenic enzymes such as SCD1 (**Figure 8**). The second observation was that Scia did not impact on the expression of FA Translocase/CD36 (FAT/CD36), which is known to facilitate long chain FA transport in hepatocytes. Scia also had no effect on transcription factors such as Liver X Receptors, Farnesoid X Receptor and the Hepatocyte Nuclear Factors 1 α and 4 α (results not shown). On the contrary, Scia slightly reduced the gene expression of peroxisome proliferator activated receptor α (PPAR α) implicated in the oxidation of FA (-32%). This effect was more pronounced when cells were cultured without FBS (-69%, not shown). Thirdly, concerning genes implied in triacylglycerol packaging, it was demonstrated that Scia reduced both ApoB (-28%) and MTTP (-49%) expression, which are especially involved in the assembly and secretion of triacylglycerol-rich very low-density lipoproteins (VLDL). Overall, these results show that Scia reduced the expression of genes regulating lipogenesis in cultured hepatocytes.

4. Discussion

In the first part of the study, Scia was unexpectedly identified in human serum and also in the different tissues of rats, both times present at a similar level (<0.40% of the total FA). Scia represents a phylogenic marker of gymnosperm plants due to its specific structure. Its occurrence in mammals was unexpected but corroborates previous results obtained in the animal kingdom i.e. in crickets [19]. Scia may be arise from the diet or *de novo* synthesized from precursors. No further analysis of the lipid structure bearing Scia in serum or tissues was done but this finding suggests a new functionality of this FA as proposed by the work of Nakane et al on cannabimimetic 2-sciadonoylglycerol [20]. Furthermore, a correlation between the presence of Scia and age of human subjects was found. This data remains to be confirmed for a broader cohort but could lead to the

definition of a new blood marker in childhood or ageing physiology and pathology. Furthermore, it was shown in cell models that this particular FA is synthesized after $\Delta 5$ -desaturation of 20:2n-6 by the $\Delta 5$ -desaturase. The metabolic pathway of 20:2n-6 has already been suggested for the liver and testes of rats [21, 22] and later evidenced on microsomes [23]. 20:2n-6 was preferentially $\Delta 5$ -desaturated to produce 20:3 $\Delta 5,11,14$ whereas synthesis of 20:3n-6 ($\Delta 8,11,14$) was not enhanced without the overexpression of $\Delta 6$ -desaturase. Indeed this enzyme is capable of $\Delta 8$ -desaturation on 20:2n-6 and 20:3n-3 [24]. Consequently, when cells lack a significant $\Delta 6$ -desaturase activity, such as K562, the synthesis of Scia is favoured over its 20:3 isomer [25]. Thus a *fads2*^{-/-} mouse mutant accumulates Scia in phospholipids by replacing, in part, arachidonic acid and other polyunsaturated FA [26]. The physiological effects of Scia were consequently attributed to this arachidonate substitution. Nevertheless Scia was also shown to lower by itself the level of triacylglycerols in plasma and liver [8, 10, 11].

In the second part of the study, the mechanism of the action of Scia in cultured hepatocytes was investigated with regard to its potential effect on SCD1. This $\Delta 9$ -desaturase is involved in the biosynthesis of monoenes, in particular 18:1n-9 and 16:1n-7. These monounsaturated FA are esterified in neosynthesized triacylglycerols that are later secreted by hepatocytes through nascent VLDL. The results showed that Scia reduced the gene and protein expression of SCD1 in cultured hepatocytes. Therefore $\Delta 9$ -desaturation of saturated FA (18:0 and 16:0) was lowered independently of the substrate level. Indeed the results showed that Scia tended to reduce the expression of *Fasn* and also significantly decreased that of *elovl6*. Nevertheless the FA profiles obtained in this study showed no significant modification of 16:0 and 18:0 proportions, independently of the culture condition. The observed effect was thus specific to SCD1 with respect to the FA profile. Scia behaved like an usual polyunsaturated FA in spite of the lack of methylene-interrupted double bond at C5. Indeed the gene expression of *Scd1* was shown to be depressed in rodents fed with fish oil enriched-chow or with a custom diet supplemented with eicosapentaenoic acid (EPA) and docosahexaenoic

acid (DHA) [27–29]. A similar result was observed *in vitro* on SCD1 protein expression of 3T3-L1 adipocytes treated with EPA, docosapentaenoic acid (DPA) and DHA as compared to 18:0 [30]. This work demonstrated that Scia behaves like these regular polyunsaturated FA despite the absence of a more typical ethylenic structure.

SCD1 activity was studied further and revealed that Scia was also able to modulate catalysis *in situ*, potentially by acting directly on the protein complex as an effector or by acting as a non-competitive inhibitor. This effect was not specific since 20:3 FA were also shown to be regulators of SCD1 activity. Nonetheless, concerning the chemical structure of 20:3 isomers, the inhibition was more pronounced when the molecule has a double bond close to the carboxylic end, such as a $\Delta 5$ -unsaturation. In cases of tissue inflammation, Scia presents the advantage to being devoid of $\Delta 8$, thus preventing the formation of eicosanoid derivatives [5–7]. In triglyceridemia, the $\Delta 5$ -unsaturation of Scia would have been the structural element implied in the regulation of SCD1 kinetics. The significance of the $\Delta 5$ occurrence was underlined in the work of Huang et al, where a difference between Scia and 20:2n-6 ($\Delta 11,14$) was observed on the production of pro-inflammatory modulators [7]. In this work, the methylene-interrupted structure of 20:3n-6 ($\Delta 8,11,14$) and 20:3n-9 ($\Delta 5,8,11$) was slightly favoured from amongst the 20:3 isomers regarding the activity of SCD1. The inhibiting effect was contrasted with 20:3n-3 ($\Delta 11,14,17$), in which the unsaturated bounds are located further from the carboxyl end of the molecule. SCD1 may be inhibited by FA in as much as FA exhibit unsaturations before C9 in the carbon chain.

By increasing the pool of monoenes in hepatocytes, SCD1 contributes to triacylglycerol neosynthesis, in which its availability regulates VLDL assembly and secretion. Its inhibition by Scia consequently reduced the secretion of triacylglycerols by hepatocytes, as previously underlined by *in vivo* studies [8, 10, 11]. *De novo* neosynthesis of triacylglycerols is regulated by SREBP1c, a hepatic gene transcription factor that stimulates the synthesis of lipogenic enzymes. It was further demonstrated that Scia strongly inhibited gene expression of *SREBP1c* as described earlier with polyunsaturated

FA .

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such as EPA [31]. Finally, Scia could be considered as a polyunsaturated FA close to the n-6 family with respect to its structure and its metabolic features like the accumulation of linoleic acid. This study underlined that this unusual FA behaved as a typical polyunsaturated FA by reducing factors involved in lipogenic pathways. In this way, Scia inhibited the $\Delta 9$ -desaturase that is considered as a key regulator of lipid homeostasis, specifically with respect to the secretion of nascent triacylglycerols. In parallel, its circulating occurrence opens up new opportunities for future study. It would be of special interest to understand if this unusual FA is actually produced by the organism or if it is preferentially derived from the diet. Scia is a distinct plant FA particularly abundant in seeds, but it is also present in leaves and woods of coniferophytes and some cycadophytes [32]. Scia may be considered as a widely present $\Delta 5$ -olefinic acid since it is consistently recovered from the analyzed seeds of gymnosperms and in particular of *Pinaceae* [33]. Along with juniperonic acid (20:4 $\Delta 5,11,14,17$), it represents the most abundant C20 polyunsaturated FA found in land plants. Interestingly, when Scia is absent from seeds, cyclopropenoic (malvalic and sterculic) FA have been described. These compounds characterized by a cyclopropene ring at C9 are known as inhibitors of SCD1 [34]. The parallel between Scia and these cyclopropene FA warrant further comment in the context of the present study. Although Scia-enriched seeds are not all edible, recipes exist in almost every culinary practice, especially with pine nuts (also named pignolas or pinyons). For instance, the Americans cook pignoli cookies, a sort of macaroon topped with pine seeds. In Europe, the Italians include pignolias as a component of the pesto sauce used to top the pasta. In the south west of France, the salade landaise is livened up with pinyons from *Pinus pinaster* that contains around 7% of Scia. Nonetheless, pine nuts do vary between Europe and Asia depending on the local resources or the origin of the import. Finally Scia-enriched seeds are included as an ingredient of food preparations around the world and may incidentally contribute to the regulation of triglyceridemia.

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This research is dedicated to Robert Wolff[†], who was FP's mentor in lipochemistry.

Author contributions

F.P. designed and supervised the study, contributed to the cell experiments, analyzed the data, and wrote the manuscript. N.B.M. organized and carried out experiments and also helped to the data analysis. F.B. carried out the work with rats (animal care and liver perfusions) and contributed to the technical support. J.O. carried out analyses on the samples of human tissue. F.L.L. illustrated the molecular structures drawn by using ChemDraw Professional 15.0 software.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Figure 1. Detection of Scia in human serum and rat tissues.

A. Human serum profiled by GC for quantification of Scia (n=11). The identification of Scia was confirmed after purification of sera by silver nitrate-TLC. Scia was identified by mass spectrum and by comparison with the standards: ¹ 20:2n-6 (Δ 11,14), ² 20:3n-9 (Δ 5,8,11), ³ 20:3 Δ 7,11,14, ⁴ 20:3n-6 (Δ 8,11,14), ⁵ 20:4n-6 (Δ 5,8,11,14), ⁶ 20:3n-3 (Δ 11,14,17). **B.** The correlation between serum Scia and the age of donors (n=11), depending on men (●) and women (○). **C.** Scia in rat tissues (mean \pm SD, n \geq 5). Scia proportion expressed as a % of the total FA is also specified besides the bars of graph. BAT: Brown Adipose Tissue, WAT: White Adipose Tissue, RBC: Red Blood Cells.

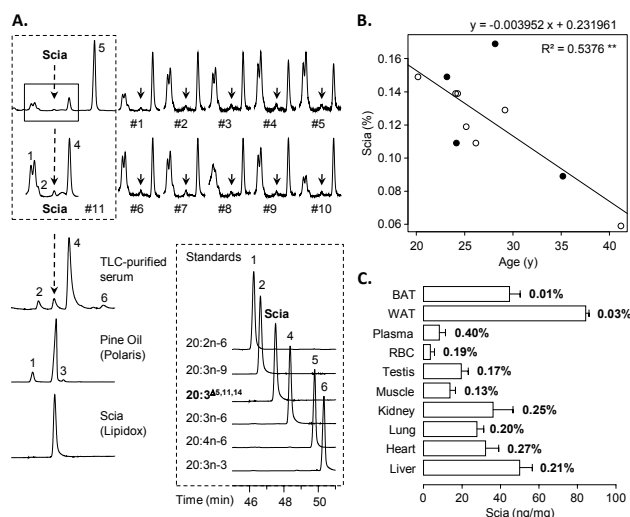


Figure 2. Scia synthesis after $\Delta 5$ -desaturation of 20:2n-6 in transfected SH-SY5Y cells and rat hepatocytes.

A-B. SH-SY5Y neuroblastoma cells were transfected with rat *Fads1*, *Fads2* or *Fads3* genes, coding for the $\Delta 5$ -desaturase, $\Delta 6$ -desaturase and FADS3 respectively. They were subsequently treated with 20:2n-6 for 24h (mean \pm SD, n=3). **A.** Results from GC analysis focused on 20:2n-6 and its derivatives for each set of culture conditions. **B.** FA profiles centered on Scia and 20:2n-6 derivatives. Results are expressed as a % of the total FA. The difference between the control and the *fads* overexpression was evaluated by a paired-t test ($p < 0.05$). **C.** Primary hepatocytes were treated for 24h with 20:2n-6. Incorporation of 20:2n-6 is given as a % of the total FA (first graph), whereas the other FA profiles are presented as a % of the total FA excluding 20:2n-6 (mean \pm SD, n=3).

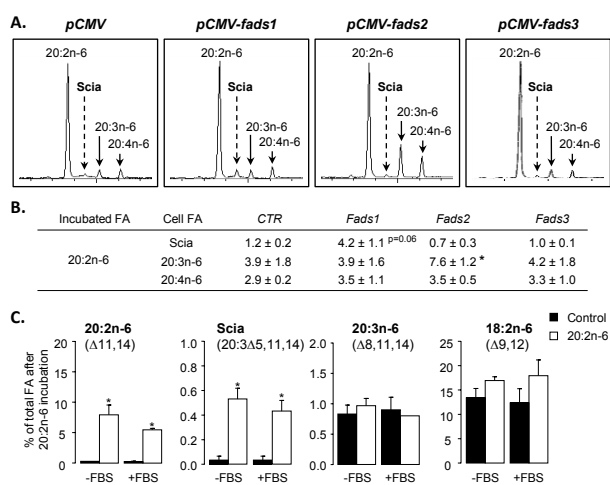


Figure 3. Incorporation of Scia into rat hepatocytes.

Hepatocytes were cultured for 24h in DMEM in the absence of FBS, with or without insulin, and were treated with Scia. **A.** The incorporation of Scia into cells. Results are expressed as a % of the total FA including Scia (mean \pm SD, n=4). The numbers on the bars indicate the amount of Scia (in nmol) per dish. Left: Scia elution with partial chromatograms of two representative samples. **B.** Scia distribution in the different compartments of cultured cells (mean \pm SEM, n=4).

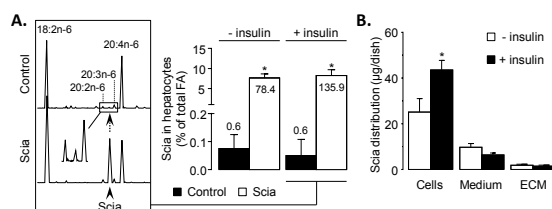


Figure 4. Metabolism of Scia and its consequences on other FA in rat hepatocytes.

Hepatocytes were cultured for 24h in DMEM in the absence of FBS, with or without insulin, and were treated with Scia. Results are expressed as a % of the total FA excluding Scia (mean±SD, n=4).

A. The metabolism of Scia in hepatocytes. The numbers on the bars indicate the amount of FA (in nmol) per dish. **B.** The impact of Scia on n-6 FA, monoenes (16:1n-7+18:1n-7+18:1n-9) and Δ 9-desaturation indexes. For monoenes, each symbol represents one experiment: differences between the control and Scia are significant when evaluated by a paired-t test ($p < 0.05$).

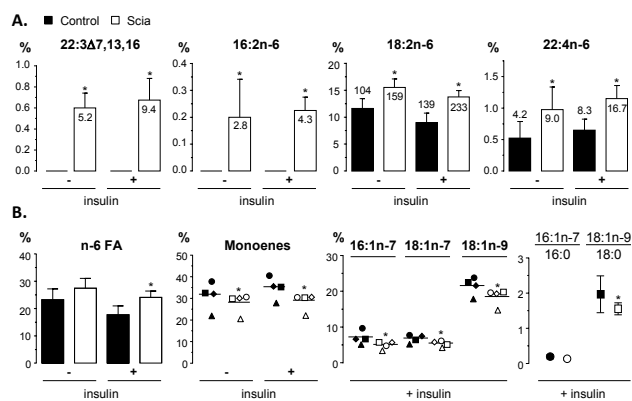


Figure 5. Modulation of the expression of lipogenesis enzymes by Scia in rat hepatocytes.

Hepatocytes were cultured for 24h in DMEM with or without FBS and insulin, and were treated with Scia. **A.** The effect of Scia on SCD1 expression (mean±SEM, n=3). **B.** The effect of Scia on mRNA expression of enzymes involved in FA biosynthesis. Hepatocytes were cultured in medium deprived of FBS and treated (□, ○) or not (■, ●) with Scia. Results are expressed in arbitrary unit from the calculation of $2^{-\Delta Ct}$ (mean±SEM, n≥3). The $\Delta 9$ -, $\Delta 5$ - and $\Delta 6$ -desaturases are encoded by *Scd1*, *Fads1* and *Fads2* respectively. *Fads3* codes for FADS3. ACC is encoded by the *Acc* gene and catalyzes the biosynthesis of malonyl-CoA from acetyl-CoA. FAS is encoded by the *Fasn* gene and is involved in *de novo* biosynthesis of 16:0 from malonyl-CoA and acetyl-CoA. Elovl6 is encoded by the *Elovl6* gene and catalyzes the elongation of 16:0 into 18:0.

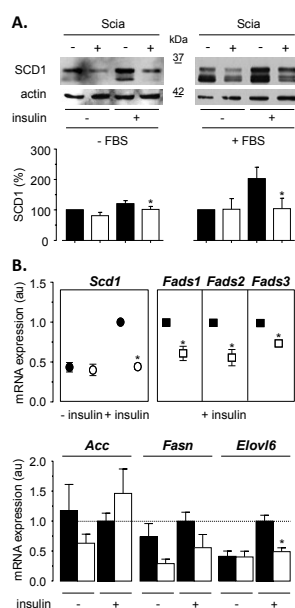


Figure 6. Reduction of secretion of triacylglycerols by Scia from rat hepatocytes.

Hepatocytes were cultured for 24h, 48h and 72h in DMEM with FBS, insulin and dexamethasone, and were treated with Scia. **A.** The effect of Scia on monoenes (16:1n-7+18:1n-7+18:1n-9) in cells. Results are expressed as a % of the control calculated from the monoene amounts expressed in μg per dish (mean \pm SEM, n=3). **B.** The expression of SCD1 as a function of time. Only one of three experiments is shown. **C.** The amount of triacylglycerol in hepatocytes and the culture medium (mean \pm SEM, n=3 in duplicates). **D.** The correlation between the amounts of triacylglycerol and 18:1n-9 in cells treated or not with Scia (mean \pm SEM, n \geq 3). The top graph shows that the correlation between triacylglycerols and 18:1n-9 is significant. In the lower graph, each point corresponds to an incubation time: the cellular triacylglycerol concentration increased gradually from 24h to 72h. **E.** Triacylglycerol staining in hepatocytes cultured for 72h with Scia. Lipid droplets were colored with oil red O and observed by microscopy (magnification 40x). **F.** Triacylglycerol synthesis and secretion estimated by [^3H]-glycerol labelled triacylglycerols in culture compartments (mean \pm SEM, n=4 in triplicates).

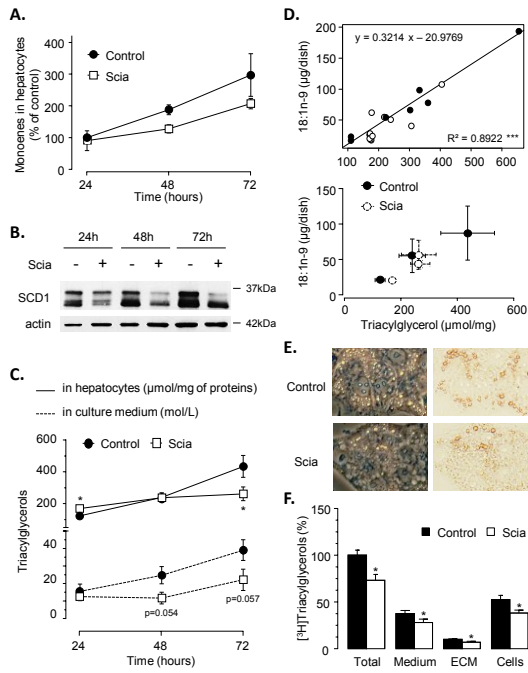


Figure 7. Reduction of the activity of SCD1 in liver by Scia and other 20:3 isomers.

Post-mitochondrial supernatant from liver was incubated for 10 minutes with 15 μ g of FA/mg of proteins or with ethanol as vehicle. The Δ 9-desaturase activity was then measured on either [14 C]-16:0 or [14 C]-18:0 for 20 minutes at 37°C. **A.** The effect of Scia on SCD1 activity measured on both substrates (mean \pm SEM, n \geq 5 in duplicates). **B.** The effect of 20:3 isomers on SCD1 activity on the 18:0 substrate (mean \pm SEM, n=4 in duplicates). Different letters between bars indicate a significant difference between FA.

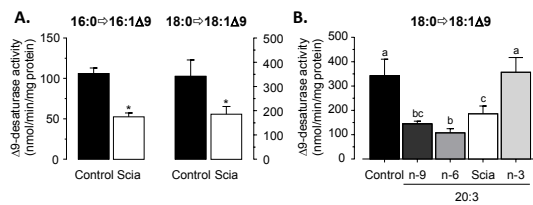
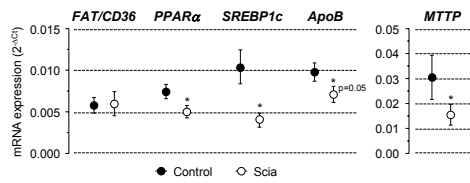


Figure 8. The effect of Scia in regulating the genes involved in lipogenesis, triacylglycerol synthesis and VLDL secretion in rat hepatocytes.

Hepatocytes were cultured for 24h in DMEM with FBS and insulin, and were treated with Scia. Gene expression was measured and expressed in $2^{-\Delta Ct}$ in reference to *gapdh* (mean \pm SEM, n=5).



Supplemental Figure 1. The structure of Δ^5 -olefinic acids (A) and Scia compared to the other 20:3 isomers (B).

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Table 1. Lipid profiles of human serum.

Cholesterol and triacylglycerols were quantified by colorimetric dosage. The FA profiles were attained by GC-MS. Polyunsaturated FA (PUFA) were calculated without Scia and the n-9 % value corresponds to monoenes, since 20:3n-9 was coeluted.

n=11	Mean ± SD	Range
Age (y)	27.2 ± 6.0	20-41
Triacylglycerol (mmol/L)	1.15 ± 1.04	0.44-4.21
Cholesterol (mmol/L)	4.69 ± 1.36	2.82-8.08
Scia (%)	0.13 ± 0.03	0.06-0.17
16:1n-7 (%)	1.7 ± 0.5	1.1-2.6
18:1n-7 (%)	1.0 ± 0.4	0.7-1.4
18:1n-9 (%)	21.9 ± 3.9	17.4-31.3
16:1n-7/16:0	0.08 ± 0.02	0.05-0.11
18:1n-9/18:0	2.45 ± 0.67	1.65-3.73
FA (µg/µL)	3.5 ± 1.8	2.6-8.9
saturated (%)	33.9 ± 1.2	31.5-36.2
monoenes (%)	26.5 ± 3.8	21.4-35.5
PUFA (%)	39.5 ± 4.2	30.6-45.8
n-6 (%)	35.1 ± 3.5	28.5-40.8
n-3 (%)	3.1 ± 0.9	1.5-4.1
n-9 (%)	22.2 ± 3.9	17.7-31.9
Σn-6 / Σn-3	11.2 ± 5.1	8.4-25.6

Supplemental Table 1. Forward and reverse primers for SYBR®Green PCR.

<i>Genes</i>	<i>Forward (5'3')</i>	<i>Reverse (5' 3')</i>
<i>ACC</i>	attgthgctcaaactgcaggt	gccaatccactcgaagacca
<i>FASN</i>	tggatagaccggtatgtcc	tcagcttccagaccgctta
<i>Elovl6</i>	agcaaagcaccgaactagg	gtacaggagcacagtgatgtgg
<i>SREBP1c</i>	cactccagctagaccccaa	ggtgagagccttgagacagt
<i>MTP</i>	ctacgtagaacgcagtcccc	ttaagcctccagcccttg
<i>ApoB</i>	catgtgtctgaagccatctg	acttggtgatacctcctctg
<i>PPARα</i>	ttcacgatgctgtcctcct	catccagtcgagggcattg
<i>FAT/CD36</i>	gttattggtgctgtcctgg	ctctgggtttgcacgca
<i>GAPDH</i>	tgtgaacggattggccgta	gatggtgatgggttcccgt