Fungal Genetics and Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Autoxidated linolenic acid inhibits aflatoxin biosynthesis in *Aspergillus flavus* via oxylipin species

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ARTICLE INFO

Article history:
Received 10 August 2014
Revised 23 November 2014
Accepted 26 November 2014
Available online xxxx

Keywords:
Linolenic acid
Autoxidation
Oxylipins
Inhibition
Aflatoxin production
Aspergillus flavus

ABSTRACT

Aflatoxins produced by *Aspergillus* species are among the most toxic and carcinogenic compounds in nature. Although it has been known for a long time that seeds with high oil content are more susceptible to aflatoxin contamination, the role of fatty acids in aflatoxin biosynthesis remains controversial. Here we demonstrate in *A. flavus* that both the saturated stearic acid (C18:0) and the polyunsaturated linolenic acid (C18:3) promoted aflatoxin production, while C18:3, but not C18:0, inhibited aflatoxin biosynthesis after exposure to air for several hours. Further experiments showed that autoxidated C18:3 promoted mycelial growth, sporulation, and kojic acid production, but inhibited the expression of genes in the AF biosynthetic gene cluster. Mass spectrometry analyses of autoxidated C18:3 fractions that were able to inhibit aflatoxin biosynthesis led to the identification of multiple oxylipin species. These results may help to clarify the role of fatty acids in aflatoxin biosynthesis, and may explain why controversial results have been obtained for fatty acids in the past.

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1. Introduction

Aflatoxins (AFs) are a group of highly toxic and carcinogenic natural compounds produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* in seeds, both pre- and post-harvest. These compounds pose serious threats to human and animal health (Amaike and Keller, 2011; Kensler et al., 2011; Roze et al., 2013). It has been known for a long time that seeds of plants such as maize, peanut, and walnut with high oil contents are more susceptible to AF production following infection by *Aspergillus* species (Fabbri et al., 1980; Reddy et al., 1992; Severns et al., 2003). It is also known that unprocessed seeds support AF production better than defatted seeds (Reddy et al., 1992; Mellon et al., 2000). Studies in maize have shown that AF accumulation is more abundant in oil-rich embryos and aleurone layers as compared to starchy endosperms (Brown et al., 1993; Keller et al., 1994). These results suggest that fatty acids (FA) are important for AF biosynthesis. However, *in vitro* experiments show that stearic acid (C18:0), oleic acid (C18:1) or linoleic acid (C18:2) supplied to sugar-containing media inhibit AF biosynthesis (Schultz and Luedecke, 1977). Pyiyadarshini and Tulpule reported that mixed FAs from peanuts and saturated FAs such as myristic acid (C14:0), palmitic acid (C16:0), and C18:0 promote, while unsaturated FAs such as C18:1 and C18:2 inhibit AF biosynthesis (Priyadarshini and Tulpule, 1980). Other studies showed that C18:1 and C18:2 had no effect on AF production (Fanelli et al., 1983). Thus, the role of FAs on AF biosynthesis remains controversial.

Oxylipins are known to play important roles in AF biosynthesis. In particular, it has been shown that 13*S*-hydroperoxy-9,11-octadecatrienoic acid (13*S*-HPODE) and 13*S*-hydroperoxy-9,11,15-octadecatrienoic acid (13*S*-HPOTE) inhibit, while 9*S*-HPODE, under certain conditions, promotes AF production (Burow et al., 1997). Deletion of the oxylipin-generating enzyme genes oxygenase B (*ppoB*) or *ppoC* in *A. nidulans* enhanced sterigmatocystin (ST, a precursor in AF biosynthesis pathway) production, while deletion of *ppoA* reduced ST production (Tsitsigiannis and Keller, 2006). In *A. flavus*, deletion of the lipoxygenases gene A*flox1* hampered HPODE production and AF biosynthesis *in vitro*. However, when the same strain was used to inoculate maize kernels, enhanced AF production was observed (Scarpari et al., 2014). These results imply a complex role of oxylipins in AF biosynthesis.

In this study, we re-visited the effects of saturated and polyunsaturated FAs on AF biosynthesis in *A. flavus*, and observed that both C18:0 and C18:3 promoted AF production. However, following exposure to air C18:3, but not C18:0, inhibited AF production. Detailed studies revealed that the inhibitory effect was generated in C18:3 after exposure to air for several hours. The inhibition occurred through suppressed expression of genes in the AF biosynthetic gene cluster, together with enhanced mycelial growth,

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http://dx.doi.org/10.1016/j.fgb.2014.11.005
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Please cite this article in press as: Yan, S., et al. Autoxidated linolenic acid inhibits aflatoxin biosynthesis in *Aspergillus flavus* via oxylipin species. Fungal Genet. Biol. (2015), http://dx.doi.org/10.1016/j.fgb.2014.11.005

sporulation, and kojic acid production. Mass spectrometry analyses of the autoxidated C18:3 fractions with inhibitory effects led to the identification of 15 oxylipin species.

2. Material and methods

2.1. Fungal strains and growth conditions

A. flavus strain A3.2890 was obtained from the China General Microbiological Culture Collection Center at the Institute of Microbiology, Chinese Academy of Sciences (Yan et al., 2012); the Papa 827 strain was provided by Prof. G.A. Payne from North Carolina State University; the NRRL 3357 strain was obtained from USDA-ARS, Peoria, Illinois. A3.2890 was used in all experiments, unless otherwise specified. To initiate cultures, spore suspensions and media were prepared as described previously (Yan et al., 2012), with an initial spore density of 10^6 spores/mL. All cultures were grown in continuous darkness, at 28 °C, either statically when cultured on plates, or on a shaker at 180 rpm when cultured in liquid media.

2.2. FA assays

C18:0 (catalog number S4751) and C18:3 (62160) were purchased from Sigma–Aldrich (St. Louis), with purities above 99% and 98.5%, respectively. One hundred-fold stock solutions were used to prepare the aliquots for the experiments; 200 μL of a stock solution was transferred to a conical flask, and these flasks were then placed either in a laminar flow cabinet or in a vacuum freeze dryer (50 mbar; ModulyoD, Thermo) for 4 h, to allow the ethanol to evaporate completely. 18 mL of liquid glucose mineral salts (GMS) medium and 2 mL of spore suspension (10^7 spores/mL) were added to each flask and cultured. For the growth measurements, mycelia cultured in liquid GMS media with 1.25 mM air-dried C18:0, C18:3, or without exogenous FAs were harvested by filtration on days 2, 3 and 4, and then dried in a freezer–drier prior to weighing. AF content in media was analyzed with thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC), as described previously (Yan et al., 2012). To examine the effects of FAs on sporulation, 5 mg C18:0 or C18:3 was dissolved in 200 μL ethanol (final concentration 25 mg/mL), spotted on 1 cm^2 filter paper discs and air-dried in a laminar flow chamber for 4 h before being placed on the surface of solid two-layer GMS media, as described previously (Tsitsigiannis and Keller, 2006). After 3 days of culturing, the top-layer medium was collected using a cell scraper, and the total numbers of spores were counted under a microscope with a haemacytometer.

2.3. Measurements of glucose, kojic acid, and NOR content

After mycelia were cultured in liquid GMS media with 1.25 mM air-dried C18:3 or C18:0, or media lacking exogenous FA for 2, 3, or 4 days, samples were collected by passing the media first through filter paper and then through a 0.22-μm hydrophilic nylon filter. The glucose content in media was measured using a glucose determination kit (APPLYGEN, Beijing). The kojic acid content was determined according to Bentley (1957). The norsolorinic acid (NOR) content was measured using solid potato-dextrose agar (PDA) media as described previously (Hua et al., 1999).

2.4. Real-time PCR analyses

Total RNA was extracted from mycelia after growth in liquid GMS media with 1.25 mM air-dried C18:3 or C18:0, or media lacking exogenous FA for 3 days, using previously described methods (Zhang et al., 2014). Poly(A) mRNA was purified using a PolyATrack mRNA Isolation Kit (Promega, Madison), and cDNA was synthesized using a ReverTra Ace-α-® Kit (Toyobo, Japan). Real-time PCR was performed using SYBR Green I mix in a Rotor-Gene 3000 Cycler (Corbett Research, Australia), with primers and cycling programs listed in Table 1. Relative expression levels were quantified by comparing the expression of target genes with the expression of *β-tubulin*.

2.5. Oxylipin analyses

Air- and vacuum-dried C18:3 samples were analyzed using a liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer (LC/Q-TOF-MS). For the activity assays, autoxidated C18:3 was first separated with semi-preparative HPLC (Agilent 1200, Waldbronn, Germany) using a reverse phase C18 column (4.6 mm × 150 mm, 5 μm, Agilent); fractions were collected each minute from the 9th to the 15th min. These fractions were dried completely under nitrogen gas and tested in *A. flavus* assays for their effect on AF production, as described above. Individual fractions were characterized further in an ultra-fast LC system (Shimadzu, Kyoto) equipped with a C18 column (3 × 150 mm, 3.5 μm, Eclipse XDB, Agilent). The mobile phase was prepared by mixing solution A (water with 5 mM ammonium acetate and 0.02% formic acid) and solution B (water: acetonitrile, 5: 95, with 5 mM ammonium acetate and 0.02% formic acid) in a multi-step linear gradient of 20% solution B at 0–0.5 min, 20–35% solution B at 0.5–60 min; 35–50% solution B at 60–90 min, 50–70% solution B at 90–95 min; and 70–100% solution B at 95–115 min and 100% solution B at 115–120 min, with a flow rate of 0.6 mL/min. The sample injection volume was 3 μL. MS/MS analyses of autoxidated C18:3 were performed with a hybrid Q-TOF-MS equipped with an ESI source (Triple TOF™ 5600+, AB SCIEX, Foster City, CA): one TOF MS survey scan was followed by 8 MS/MS scans. The mass range for the MS scan was set to m/z 100–600. For the MS/MS scans, the range was set at m/z 50–600. Instrumental conditions for the MS/MS experiments were: ion spray voltage, −4500 V; ion source gas 1 (nebulizer gas), 55 psi; ion source gas 2 (heater gas), 55 psi; temperature, 550 °C; curtain gas, 30 psi;

Table 1
List of primers and PCR cycling programs used in this study.

Genes	Primers used	PCR amplification schemes
aflO	F: GTCGCATATGCCCCGGTCGG R: GGCAACCAGTCGGGTTCCGG	94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles
aflR	F: AGCACCCTGTCTTCCCTAA R: CTGGTCTTCCTCATCCACA	94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles
aflS	F: CGAGTCGCTCAGGCGCTCAA R: GCTCAGACTGACCGCCGCTC	94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles
β-tubulin	F: CCAAGAACATGATGGCTGCT R: CTTGAAGAGCTCCTGGATGG	94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles
cypA	F: CGGCGGTCCCTCTTTTCCCG R: TGCGTCGTGAGGTCGGAGGT	94 °C, 30 s, (94 °C, 30 s; 62.5 °C, 30 s; 72 °C, 30 s) for 40 cycles
ordA	F: CAACGACGCGCAGCTGGGTA R: TCATGCCCCACCCCGCCATA	94 °C, 30 s, (94 °C, 30 s; 62.5 °C, 30 s; 72 °C, 30 s) for 40 cycles

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