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Relationship between lipids and aflatoxin biosynthesis

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Abstract

This paper describes the key role of lipids on fungal growth and of lipoperoxidation on the output of aflatoxin biosynthesis both 'in vitro' and 'in vivo'. 'In vitro' BHA, BHT and cysteamine, depending their concentration, are capable of reducing or blocking aflatoxin output induced by lipoperoxides or halomethanes in cultures of *Aspergillus flavus* or *A. parasiticus* without affecting fungal growth. 'In vivo' BHA and BHT significantly reduced aflatoxin production on wheat, maize and sunflower inoculated with aflatoxigenic *Aspergilli* essentially by preventing fungal growth. 'In vivo' the seeds surface lipids represent a very important carbon source for fungal growth.

Aflatoxin production *in vitro*

Many reviews are available about aflatoxin biosynthesis and metabolism [2, 3, 17, 19, 28, 33]. However, the role of lipids as factors related to aflatoxin biosynthesis is still debated. Our studies started some years ago from the observation that aflatoxin output was normally much higher in oily than in starchy seeds contaminated by different aflatoxigenic strains of *Aspergillus parasiticus* [6].

In vitro experiments carried out to investigate the relationship between lipids (synthetic medium supplemented with different saturated and unsaturated free fatty acids or triglycerides) and aflatoxin biosynthesis showed: saturated free fatty acids (FFA) from lauric (C_{12}) to behenic (C_{22}) support fungal growth very well without affecting aflatoxin production. Caprylic (C_8), nonanoic (C_9) and undecanoic acid (C_{11}) are toxic at concentrations higher than 0.2% w/v. 2) The higher the level of unsaturation in FFA, the higher the inhibiting effect of fungal growth (arachidonic and linolenic acids > linoleic acid > oileic acid). 3) Unsaturated triglycerides (TG) are much less toxic than the corresponding FFA. 4) The production of aflatoxins is directly related to the number of double bounds of free and esterified fatty acids: linoleic acid and trilinolein stimulate aflatoxin output more than oleic acid, triolein, and other saturated FFA and TG. It is well known that unsaterated lipids undergo a complex phenomenon of lipoperoxidation caused by different chemical-physical factors (i.e. radiatons, humidity, aging, temperature, chelating agents, antioxidants etc.) acting on the double bounds and producing toxic compounds such as lipoperoxides.

After the beginning of lipoperoxidation a free radical mechanism proceeds. Lipid hydroperoxides are unstable and spontaneously decom-

pose to form breakdown products (aldehydes, ketones, alkanes, carboxylic acids and polymerization products [31].

Towsend *et al.* [30] have reported that caproic acid (hexanoate) is a starter unit in polyketide biosynthesis. The authors obtained intact incorporation of ^{13}C-hexanoic acid into averufin (a key intermediate in the patways of aflatoxin biosynthesis) by using an averufin-accumulating mutant. They suggest that an intact hexanoate starter unit may arise from a separate synthase or from β-oxidation. A more evident stimulating effect on aflatoxin biosynthesis was obtained when we supplemented the synthetic medium with epoxides [9]. However, it must be pointed out that all these compounds, to a limited extent, arise during the lipid peroxidation of unsaturated lipids which primarily produces lipoperoxides [15]. In fact, synthetic hydroperoxides [7, 20], UV-peroxidated sterols and UV-peroxidated FFA and TG [13] added to culture media inoculated with toxigenic fungi stimulated the aflatoxin biosynthesis over two hundred times, without significantly affecting fungal growth. Also aldehydes (hexanal, benzaldehyde, valeric aldehyde) induce the aflatoxin output in liquid culture media (unpublished data). In addition, aflatoxins were produced by toxigenic *Aspergilli* during the aging process (late growth phase) and the lipoperoxidation is closely connected with the phenomenon of senescence in cells. For this reason we hypothesized that intracellular lipoperoxidation could be one of the most important factors involved with the aflatoxin biosynthesis. We used substrates capable of inducing endogenous lipoperoxidation of internal membranes of fungi and verified their effect on aflatoxin biosynthesis. We used, in particular, carbon tetrachloride (CCl_4) a well studied hepototoxin that is reported to induce peroxidation of lipid of the endoplasmic reticulum of liver cells by highly reactive trichloromethyl radicals ($CCl_3\cdot$) formed by the interaction of CCl_4 with the cytochrome P-450 (25). Bhatnagar *et al.* [1] reported the presence of cytochrome P-450 in *Aspergillus parasiticus* microsomes. The addition of CCl_4 in cultures of *A. parasiticus* and *A. flavus* highly stimulated the aflatoxin biosynthesis [10]. The presence in culture medium of phenobarbital that promotes endoplasmic reticulum biosynthesis of protein and phospholipids induced all the components of NADPH-cytochrome P-450 to stimulate the metabolism of CCl_4 and consequently the aflatoxin output by lipoperoxidation induced by $CCl_3\cdot$ on unsaturated lipids of microsomal and probably mitochondrial membranes [20].

Similar results were obtained with different halomethanes and organic solvents [11, 21]. It is interesting to note that the stimulating effect obtained by epoxides, hydroperoxides and CCl_4 enhanced not only aflatoxin biosynthesis but their well known congeners [14]. Our results have shown that the stimulating effect of lipoperoxidation would appear to take place before the biosynthesis of norsolorinic acid, the first known stable precursor of aflatoxin biosynthesis.

These results lead us to consider lipoperoxidation as a key factor in aflatoxin output. Unfortunately, no effective techniques are available at present for a direct detection of lipoperoxides in cells. We failed to demonstrate the lipoperoxidation induced by CCl_4 in microsomes of *A. parasiticus* by the thiobarbituric (TBA) test [22]. The TBA test is commonly used to indicate the presence of malondialdehyde, the main breakdown product of lipoperoxidation of polyunsaturated fatty acids (i.e. mainly arachidonic acid). The absence of this polyunsaturated fatty acid in the lipid pattern of aflatoxigenic fungi [8] is one of the reasons of the failure to demonstrate the endogenous membrane lipoperoxidation with the TBA test. On the other hand, by-products of lipoperoxidation, such as malondialdehyde and other aldehydes may be inactivated by reacting with $-SH$ and $-NH_2$ groups of aminoacids, so that the reaction would not take place. For this reason the problem was approached differently. We extracted lipids of microsomes and mitochondrial fractions by using methods which did not permit the occurrence of lipid peroxidation during the work-up procedures. The fatty acid patterns of purified fractions, i.e. phospholipids (PL), TG, FFA and sterols esters (SE) were analysed. The unsaturated/saturated fatty acids

ratios of PL, TG, FFA and SE were analysed. The unsaturated/saturated fatty acids ratios of SE, TG, FFA and PL fractions of lipids extracts of *A. parasiticus* appeared fairly constant when the toxigenic fungus grew for 10–15 days in synthetic medium (Czapek liquid medium). On the contrary, the ratios fall drastically in PL of microsomes and partially of the mitochondria of 15-day-old *A. parasiticus* grown on Czapek liquid medium supplemented with CCl_4 [22]. These results indicate that lipoperoxidation takes place. In addition, peroxidative decomposition of structural lipids led to a loss of microsomal enzymatic activities, i.e. cytochrome P-450, NADPH cytochrome C reductase and aminopyrine demethylase [22].

In the same paper we have reported that if CCl_4 induced an aflatoxin biosynthesis stimulation mediated by mixed function oxidase activity (MMFOA), on the contrary, the increased production of aflatoxins induced by linoleic acid hydroperoxide does not appear to be mediated by MMFOA. This differential action is still under investigation.

Very recently [24], it has been reported that several species of fungi belonging to the genera *Aspergillus, Penicillium, Fusarium* utilizing FFA such caprylic, palmitic and oleic acids modify the lipid pattern of their mycelia with the presence of polyunsaturated fatty acids (i.e. arachidonic and linolenic acids). This might open new perspectives of research about this question: how does lipoperoxide work during the induction of aflatoxin biosynthesis? Moreover, the presence of polyunsaturated lipids in membranes of aflatoxigenic strains of *Aspergillus* could excite the peroxidation process and facilitate further research on the mechanism of aflatoxin production.

Control of aflatoxin production *in vitro*

If our hypothesis on the effect of lipoperoxidation is well grounded, common antioxidants and free radical scavengers such as cysteamine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), cysteine, reduced glutathione, vitamin C and vitamin E should inhibit the stimulating effect on aflatoxin output induced by CCl_4. In fact, in rate liver cells it is reported that all the antioxidants inhibit lipoperoxidation induced by CCl_4 [25, 26, 39, 32]. But in liver, CCl_4 is rapidly metabolized and microsomal lipid peroxidation takes place after a few minutes following intragastric administration. Since antioxidants are administrated simultaneously with CCl_4 or few minutes before CCl_4, it is clear that the concentration of these antioxidants is capable of scavenging $CCl_4\cdot$ radicals and damages related to microsomal lipid peroxidation such as protein synthesis and enzymatic activities.

On the contrary, the rate of CCl_4 metabolism by *Aspergilli* is much slower as compared to those described for liver. Halomethanes settled at the bottom of culture flasks and only little amount, depending upon their solubility in water, interact with the cells of *Aspergilli.* The peroxidative process persists for a long time until halomethane has been fully metabolized. During this period the antioxidants placed in the culture medium disappear giving rise to reactive by products. In our experimental conditions the antioxidants used showed surprising results. Cysteine, reduced glutathione, vitamin C and vitamin E not only did not inhibit aflatoxin biosynthesis induced by CCl_4 but further increased aflatoxin production significantly as compared to cultures supplemented with CCl_4 alone [12]. This phenomenon might be ascribed to the formation in cultures of more or less reactive species of radicals, whose effects are synergic with that of CCl_3. Conversely, cysteamine, BHA and BHT inhibited aflatoxin production induced by the halomethanes. The cysteaminyl radical which is produced during the scavenger action, did not show a prooxidant effect on $CCl_3\cdot$, probably because it preferentially underwent a reaction in combination with another cysteaminyl radical forming unreactive cystamine [13]. A similar mechanism might explain the inhibitory action of BHA and BHT. The phenoxyradicals, stable because of sterical hinderance due to the electron donor terbutyl groups in the ortho position, have a preferential bent for reacting with another phenoxyradical or per-

oxyradicals with production of unreactive compounds [12].

Control of aflatoxin production *in vivo*

In vitro lipoperoxides play a key role in aflatoxin biosynthesis as do *in vivo* naturally occurring lipoperoxides in aged and stored seeds, which induce a stimulating effect on aflatoxin biosynthesis. In fact, on different kinds of seeds we confirmed that the production of aflatoxins was much higher when *A. flavus* or *A. parasiticus* grew on oily (sunflower, arachids, soybean) than on starchy seeds (wheat, maize) [6] and we found that such output of aflatoxins was directly related to the peroxide number of lipids extracts of seeds [20]. However, it must be pointed out that in our first experiments we used sterilized seeds in studying the aflatoxin output *in vivo* induced by *A. flavus* and *A. parasiticus*. In general, the aflatoxin production on sterilized seeds infected by *Aspergilli* is much higher than in non-sterilized one. With sterilization by autoclaving biological, chemical, physical damages are produced on the seeds and many natural defence mechanisms of seeds are damaged. In addition, traces of elements, particularly zinc and phosphorous become available for the development of the *Aspergilli* because of the splitting of the bond of phytate [18]. Non-sterilized seeds represent a more truthful representation of naturally occurring mould colonization of seeds. With different non-sterilized seeds (sunflower, maize, wheat) we have studied the effect *in vivo* of different antioxidants that *in vitro* had showed a significant effect in inhibiting aflatoxin production [12]. In just-harvested oily and starchy seeds treated with antioxidants and inoculated with *A. parasiticus* the effect of some antioxidants such as BHA and BHT was evident by inhibiting fungal growth, differently from their action *in vitro* where BHA and BHT inhibit aflatoxin production without significantly affecting fungal growth. In our opinion, the different effect of BHA and BHT *in vitro* and *in vivo* experiments is due to the fact that *in vitro* BHA and BHT were added after some days after the inoculum when the fungus is in the stationary phase and the number of cells is much higher than the number of fungal conidia used as inoculum *in vivo* where BHA and BHT were added with the inoculum.

One important aspect of the subject, which as not been duly considered, is the stability of the antioxidants added to the seeds. The rate of decomposition of antioxidants, which is affected by different factors such as moisture and aging, is very different [23]. The drier and younger the seeds, the lower the rate of decomposition. It is clear that following the disappearance of BHA and BHT from the seed surface (which depends upon the initial concentration) the fungus can give rise to new growth.

It would appear that *in vivo* lipoperoxidation does not play a key role in aflatoxin output, but the following consideration must be emphasized: 1) *in vivo* the aflatoxin production in rancid seeds inoculated with *A. parasiticus* paralleled the peroxide number [20]; 2) fungal growth in oily seeds is inhibited when the concentration of superficial seed lipid weight is lower than 0.15%. If we add saturated, monounsaturated or diunsaturated TG to a seeds with concentration lower than 0.15% aflatoxin production is detected only on samples on which we added diunsaturated TG [4]; 3) the addition of UV peroxidated TG to seeds enhanced both the fungal growth and aflatoxin production [4].

As a matter of fact, lipoperoxidation is a complex phenomenon which depends upon several physicochemical factors in the environment. The peroxidation and the relevant production of organic free radicals of seeds lipids lead to a loss of viability of stored and aged seeds. Following deterioration of seeds, their enzymatic defence systems become altered and *Aspergilli* can develop more easily [23].

Conclusions

Many papers have reported a relationship between lipid metabolism and aflatoxin biosynthesis. Detroy and Hesseltine [5] reported an inverse relationship between fatty acids and afla-

toxin biosynthesis. Shih and Marth [27] reported that the addition of sodium azide, an inhibitor of terminal electron transfer, caused an increase in both aflatoxin and lipid synthesis by *A. parasiticus* due to the inhibition of oxidative respiration. They concluded that the accumulation of acetate and NADPH as bioregulators of aflatoxin biosynthesis is unclear. Gupta *et al.* [16] showed that during the stationary growth phase, zinc supplementation resulted in high adenosine-5-monophosphate (AMP) levels which have been shown to the inhibitory for aflatoxin biosynthesis.

A recent review has reported the compounds affecting the biosynthesis or bioregulation of aflatoxins [33] and as the authors have indicated most of the papers have only dealt with fungal growth and/or aflatoxin production and relatively few studies attempted to identify possible mechanisms of action. In addition many studies are *in vitro* and provide valuable data that can't be verified *in vivo*. Our experience has shown that both *in vitro* and *in vivo* lipoperoxides from any sources (synthetic and naturally occurring lipoperoxides in old oil-bearing seeds) play an important role in inducing aflatoxin production.

BHA and BHT can be considered extremely useful tools in the fight against aflatoxins in agricultural crops. In fact, they couple the capacity of inhibiting the fungal growth of aflatoxigenic fungi to their power which allows them to prevent accumulation of aflatoxin stimulation lipoperoxides. However, it must be pointed out that BHA, BHT as well vitamin E are capable of preventing, but not eliminating or lowering the levels of pre-existent lipoperoxides, when added to rancid seeds [23].

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