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Original Research Article

COMPARATIVE STUDY ON EFFECT OF COMBINATORIAL IRON-CHELATION AND OXIDATIVE STRESS ON THE GROWTH OF ASPERGILLUS SPECIES

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Abstract

Aspergillus fumigatus has to cope with a combination of several stress types while colonizing the human body. A functional interplay between these different stress responses can increase the chances of survival for this opportunistic human pathogen during the invasion of its host. In this study, we shed light on how the H2O2-induced oxidative stress response depends on the iron available to this filamentous fungus, using transcriptomic analysis, proteomic profiles, and growth assays

Introduction

Aspergillus fumigatus is a ubiquitous fungal species, which occurs commonly on decaying organic matter and in soil under a wide variety of conditions [1, 2]. This mould is also known as one of the most important airborne human pathogenic fungi with an outstandingly high mortality rate (50–95%) in im- munocompromised patients, who suffer from an in- vasive *A. fumigatus* infection (referred to as invasive aspergillosis) [3–6]. The reasons for the unique suc- cess of *A. fumigatus* as the most important oppor- tunistic human pathogen among phylogenetically closely related aspergilli are largely unknown. Several fungal factors that determine the outcome of infections have been identified [6–10]. These include the rodlet and cell wall melanin layers of conidia [11, 12]; the cell wall exopolysaccharide galactosaminoga- lactan, which has possible anti-inflammatory effects [13]; the production of mycotoxins such as the immune re- sponse modulator gliotoxin [14]; elastinolytic proteases [15]; efficient iron and zinc acquisition systems [16, 17]; acquisition and detoxification of copper [18, 19]; as well as the suitable oxidative stress defense systems to detoxify re- active oxygen species (ROS) generated by macrophages and neutrophils [10, 20]. The stress responses of *A. fumigatus* have also been studied intensively in order to identify a potential Achilles' heel of this pathogen. Researchers aimed to elucidate the orchestration of the signaling network regulating the stress response and understanding

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the physiological background of the adaptation process [21–29]. Iron starvation and oxidative stress are typ- ical stresses for *A. fumigatus* and also for other mi- croorganisms, which they may encounter in the human host [29]. *A. fumigatus* acquires iron by low affinity iron transporters, the reductive iron assimila- tion (RIA) system, and siderophore-mediated iron up- take [30]. It is unable to utilize iron directly from human iron-binding proteins like hemoglobin, trans- ferrin, or ferritin [30], however siderophores can che- late iron from host proteins [31]. The significance of the low affinity iron transport has not been studied in details so far [29, 30].

Materials and Methods

This study was conducted in the Department of Microbiology, Rama Medical College Hospital & Research Centre .*A. fumigatus* strain Af293 (CBS 101355), received from the CBS-KNAW culture collection , was used throughout this study with exception of the oxidative stress resistance analysis and was maintained on Barrat's minimal nitrate agar plates.Plates were incubated at 37 °C for 5 d. Conidia obtained from the 5-days-old cultures were used in these experiments. Modified Barrat's minimal nitrate broths (100 ml in 500 ml flasks) were inoculated with freshly isolated conidia and were incubated at 37 °C and 3.7 Hz shaking frequency. The modified Barrat's minimal nitrate broth differed from the standard Barrat's minimal nitrate medium in the added trace element solution, which did not contain EDTA and iron. This modified form of Barrat's minimal nitrate broth was either supplemented with 30 μM $FeCl_3$ (+Fe; iron-replete cultures) or used without addition of any iron (-Fe; iron-depleted cultures). Cultures (+Fe and -Fe) were inoculated with 4×10^8 and 8×10^8 conidia and were incubated for 33 h and 50 h, respectively, before harvest. At the two time points, the +Fe and -Fe cultures showed similar residual glucose content as well as similar dry cell mass. Oxidative stress was induced by addition of H_2O_2 (at 3 mM final concentration) to the cultures 1 h before sampling ($+H_2O_2$ and $-H_2O_2$ cultures).

Results and Discussion

The iron-chelation tolerance (MIC_{DFP}) varied between 0.9 mM,and 3.6 mM depending on the strain (Table 1). The mean MIC_{DFP} of strains with "large" conidia (conidial diameter >3 mm; MIC_{DFP} ¼ 3.2 ± 0.9 mM) was significantly higher than that of the strains with "small" conidia (conidial diameter <3 mm;

MIC_{DFP} 1.3 ± 0.4 mM) (Student's t-test; *p* 0.0015). When *A. fumigatus* Af293 and *Aspergillus flavus* CBS 128202 were maintained under "iron-limited conditions" (*i.e.*, they formed conidia on DFP containing media), their conidia showed reduced DFP tolerance (Table 2). This is in line with the results of Kang et al. [31] who found that the environment of sporulating *A. fumigatus* cultures determines the properties of the germi- nating spores including their virulence. Our results can be easily explained by that conidiogenic hyphae help the accumulation of iron in the conidia formed [32] to aid their germination. On DFP containing plates, it was not efficient enough and the smaller iron stores of conidia reduced their success to survive under iron limited conditions. Our data suggest that species with "large", rather than "small", conidia may have a better chance of colo- nizing iron-limited habitats like the human body. However, small conidia have a much better chance of avoiding mucociliary clearance and reaching pulmonary alveoli. Therefore, there must be an optimal range for iron-limitation

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stress tolerance depending on conidial size that is the most optimal to colonize the human lung

Table 1
Deferiprone (DFP) tolerance of different *Aspergillus* strains.

Strains	MIC^{a}_{DFP} (mM)	Relative growth in the presence of H_2O_2 (%)[b]		Conidium diameter[c] (μm)
		"conidium"	"mycelium"	
Aspergillus terreus NCCB IH2624	1.35[i]	38 ± 5[i]	45 ± 5[i]	1.9 ± 0.3[d]
Aspergillus fumigatus Af293[j]	1.80	57 ± 5[i]	94 ± 5	2.2 ± 0.3[d,e]
Aspergillus fumigatus SZMC3100[j]	0.90	82 ± 6[i]	97 ± 3	2.0 ± 0.2[d,e]
Aspergillus fumigatus SZMC3102[j]	0.90	88 ± 4[i]	85 ± 5[i]	2.0 ± 0.3[d,e]
Aspergillus fumigatus SZMC3104[j]	0.90	78 ± 3[i]	97 ± 4	2.0 ± 0.2[d,e]
Aspergillus fumigatus F.00056[j]	1.80	55 ± 6[i]	64 ± 3[i]	2.2 ± 0.2[d,e]
Aspergillus fumigatus F.00948[j]	1.35	32 ± 8[i]	48 ± 8[i]	2.0 ± 0.2[d,e]
Aspergillus fischeri CBS 544.65	1.35	99 ± 3	93 ± 5	2.6 ± 0.2[e]
Aspergillus nidulans FGSC A4	1.35	56 ± 8[i]	80 ± 6[i]	3.2 ± 0.2[f]
Aspergillus niger CBS 113.46	3.60	98 ± 2	92 ± 5	3.6 ± 0.3[f,g]
Aspergillus brasiliensis CBS 101.740	3.60	85 ± 6[i]	86 ± 4[i]	3.8 ± 0.5[f,g]
Aspergillus tubingensis CBS 134.48	3.60	85 ± 3[i]	97 ± 2	3.9 ± 0.4[g]
Aspergillus flavus CBS 128202	3.60	72 ± 3[i]	98 ± 3	4.0 ± 0.4[g]
Aspergillus oryzae Rib40	3.60	75 ± 4[i]	91 ± 3	4.8 ± 0.7[h]

Table 2
Changes in the deferiprone (DFP) tolerance of different *Aspergillus* strains in the presence of H_2O_2.

Species	MIC_{DFP} (mM)[a]						
	H_2O_2 concentration (mM):						
	0	0.63	1.25	2.5	0.63	1.25	2.5
		"Pretreated with H_2O_2"			"Pretreated with DFP"		
Aspergillus terreus NCCB IH2624	1.35	1.35	0.90	0.45	0.90	0.45	0.45
Aspergillus fumigatus Af293	1.80	2.25	2.25	0.90	1.35	0.90	0.45
Aspergillus fumigatus Af293 (DFP)[b]	0.90	1.35	1.35	0.45	0.90	0.45	0.00
Aspergillus fumigatus SZMC3100	0.90	1.35	1.80	1.80	0.90	0.90	0.90
Aspergillus fumigatus SZMC3102	0.90	1.35	1.35	1.35	0.90	0.90	0.45
Aspergillus fumigatus SZMC3104	0.90	1.80	1.80	0.45	0.90	0.90	0.90
Aspergillus fumigatus F.00056	1.35	1.80	1.80	0.45	0.90	0.45	0.45
Aspergillus fumigatus F.00948	1.35	1.35	1.35	0.45	1.35	0.90	0.45
Aspergillus fischeri CBS 544.65	1.35	1.35	1.35	1.35	1.35	1.35	1.35
Aspergillus nidulans FGSC A4	1.35	1.80	1.80	1.35	0.90	0.90	0.90
Aspergillus niger CBS 113.46	3.60	3.60	3.60	3.60	3.60	3.60	3.60
Aspergillus brasiliensis CBS 101.740	3.60	>3.60	>3.60	3.60	1.35	1.80	1.80
Aspergillus tubingensis CBS 134.48	3.60	>3.60	>3.60	>3.60	1.80	1.80	1.80
Aspergillus flavus CBS 128202	3.60	>3.60	>3.60	>3.60	3.60	2.25	1.35
Aspergillus flavus CBS 128202 (DFP)[b]	1.80	2.25	2.25	1.35	1.80	1.35	0.90
Aspergillus oryzae Rib40	3.60	>3.60	>3.60	>3.60	3.60	2.25	1.35

Importantly, H_2O_2 had no strong growth inhibitory effect even at 2.5 mM final concentration (Table 1). Despite of this observation, the applied H_2O_2 treatment markedly modified the DFP tolerance of the strains (Table 2). The outcome of the experiments highly depended on how the two stressors (DFP and H_2O_2) were combined. If conidia that had already germinated in the presence of DFP were exposed to H_2O_2, the MIC_{DFP} values generally decreased (Table 2). In this case, mycelia suffering from iron (and probably other metal ion [33] limitation were treated with oxidative stress ("pretreated with DFP"). The decreasing MIC_{DFP} values suggest that even low iron-chelation stress could enhance the growth inhibitory effect of H_2O_2. Oxidative stress sensitivity of iron-limited *A. fumigatus* cultures is a well-documented phenomenon [34]. Our data demonstrate that this phenomenon can be general within the *Aspergillus* genus which concurs well with the strategy of mammalian immune systems combining iron withdrawal with oxidative attack to suppress microbial growth. Moreover, the antifungal effect of iron chelators can be higher *in vivo* e where iron-chelation stress may combine with oxidative stress e than that based on *in vitro* studies. When H_2O_2 (final concentration 0.65 mM or 1.25 mM) was added together with DFP to the conidia, it sur- prisingly increased the MIC_{DFP} in most strains (Table 2). In this case, germinating conidia had to cope with oxidative stress relying on their own iron storages, and the negative consequences of iron-

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chelation stress was developed only following that ("pretreated with H_2O_2"). This observation suggests that adaptation to oxidative stress can counterbalance the growth inhibitory effect of iron- chelation stress. There are several data in the literature indicating that oxidative stress, depending on its type and strength, can either enhance or reduce iron acquisition [35,36]. The upregulation of iron uptake by oxidative stress could explain our results. This behavior suggests that inhaled conidia surviving the oxidative attack of alveolar macrophages may have better chance to germinate and penetrate into the lung tissue, despite of the iron-limited conditions occurring there. Actually, this may represent an Achilles' heel of the combined iron withdrawal e oxidative attack strategy of our immune system. However, making the iron-limitation stress stronger by applying iron-chelators as antifungal adjuvants may reduce the risk of the successful invasion of the host. Interestingly, those strains, where the growth inhibitory effect of H_2O_2 on conidia was strong (*Aspergillus terreus* NCCB IH2624, and *A. fumigatus* F.00948) or weak (*Aspergillus fischeri* CBS 544.65, and *Aspergillus niger* CBS 113.46) (Table 1), did not show this phenomenon (Table 2). It raises the possibility that if the oxidative stress toler- ance is too weak or too strong, oxidative stress is unable to initiate the appropriate changes in the physiology of germinating conidia to enhance their iron-limitation stress tolerance.

Conclusion

our results suggest that opportunistic human pathogens not necessarily have superior stress tolerance attributes. Any stress tolerance that is within an appropriate range (i.e. neither too strong nor too weak) may support pathogenicity. Our results also confirm the validity of antifungal approaches based on iron- chelating agents.

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