Genetics and Molecular Biology, 28, 4, 798-803 (2005)

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Research Article

Genotoxic evaluation of sodium nitroprusside in *Aspergillus nidulans*

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Abstract

The exogenous nitric oxide donor, sodium nitroprusside, evaluated the recombinogenic potential of nitric oxide. Drug inhibited mycelial growth and conidiation in A757 *Aspergillus nidulans* master strain. Two heterozygous diploid strains, one wild (*uvsH*$^+$*//uvsH*$^+$) and the other defective to DNA repair (*uvsH//uvsH*) were used for recombinagenesis tests. Sodium nitroprusside recombinogenic effect was evaluated by the induction of homozygosis of recessive genes, originally present in heterozygous condition. Results show that sodium nitroprusside (40 μM, 80 μM and 160 μM) is effective in inducing mitotic crossing-over in diploid cells of *A. nidulans.*

Key words: nitric oxide, parasexual cycle, homozygotization index, conidiation, mitotic recombination.

Received: September 24, 2004; Accepted: March 22, 2005.

Introduction

The loss of a functional copy of a heterozygous tumor suppressor gene represents an important step during neoplastic transformation. Considered as a somatic event, loss of heterozygosity (LOH) of the long arm of chromosome 13 is the most common mechanism by which the wild-type allele at the retinoblastoma tumor-suppressor locus (*RB1*) is lost in a heterozygous retinal cell for a null mutation. Retinoblastoma may arise from the resulting daughter cell which will be either homozygous or hemizygous for the mutant allele (Hagstrom and Dryja, 1999; Ramel *et al*., 1996).

Several pathways, including chromosomal deletion, mitotic non-disjunction and mitotic recombination, may lead to LOH (Petek *et al.*, 2003; Lasko *et al.*, 1991). Mitotic recombination consists of exchange events between homologous chromosomes that, following chromosome segregation and cell division, may result in homozygosis of distal genes to the point of exchange (Biggins and Murray, 1999; Beumer *et al.*, 1998). This process plays an essential role in the DNA repair mechanism of eukaryotic cells (Seoighe and Wolfe, 1998; Wolfe and Shields, 1997) and may be initiated by double- or single-strand breaks (Biggins and Murray, 1999; Galli and Schiestl, 1998; Geigl and Eckardt-Sxhupp, 1991).

Although the genetic control of mitotic recombination is poorly understood, it is known to increase in eukaryotic cells in response to a variety of DNA-damaging agents such as ionizing and non-ionizing radiation and chemical substances (Becker *et al*., 2003; Geigl and Eckardt-Schupp, 1991; Takabayashi *et al*., l984). Intercalating agents such as cryptolepine that induce breaks in DNA and inhibitors of DNA synthesis such as danofloxacin are in fact known as recombinagenic effectors (Chiuchetta and Castro-Prado, 2002; Leonardo and Castro-Prado, 2001).

The free radical gas nitric oxide (NO) acts as a transcellular messenger molecule in both physiological and pathological processes in humans, including inflammation and cancer (Lala and Orucevic, 1998; Moncada *et al*., 1991; Collier and Vallance, 1989). The gaseous molecule is synthesized by nitric oxide synthase (NOS) from L-arginine (L-Arg) and has been shown to have tumor promoting and inhibitory effects (Xu *et al*., 2002; Brennan and Moncada, 2002). In the intracellular environment nitric oxide is a highly reactive molecule causing DNA damage via the generation of peroxynitrite (ONOO-), which can cause DNA single-strand breaks, and nitrogen trioxide (N_2O_3), which can cause DNA-crosslinking. One of the consequences of nitric oxide mediated DNA damage is to trigger accumulation of the p53 which may lead to cell cycle arrest and cellular apoptosis, this being a possible pathway by which nitric oxide may exert anti-tumor properties (Xu *et al*., 2002; Forrester *et al*., 1996; Wink *et al*., 1991). Alternatively, nitric oxide generated by NOS may stimulate tumor growth and metastasis by promoting new blood vessel formation through the up-regulation of vascular endothelial growth factor (VEGF), a glycoprotein that causes increased mitosis in endothelial cells (Lala and Chakraborty, 2001; Moochhala and Rajnakova, 1999).

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Sodium nitroprusside (SN) is a nitric oxide donor which can cause DNA single-strand breaks resulting in mitotic crossing-over. This chemical was recently suggested as a treatment for cerebral ischemia in patients with severe, medically refractory vasospasm after subarachnoid hemorrhage (Raabe *et al.*, 2002). Vasodilating therapy with sodium nitroprusside has also proven effective in patients with left ventricle failure, but arterial hypotension is its main side effect (Bregagnollo *et al*., 1999).

The filamentous fungus *Aspergillus nidulans* has often been used in mitotic crossing-over studies because this fungus spends a substantial proportion of its life cycle in the G2 phase (Bergen and Morris, 1983). Our research investigated the recombinogenic potential of the nitric oxide donor sodium nitroprusside in diploid *Aspergillus nidulans*. strains

Material and Methods

Fungal strains and culture media

The origin and the genotypes of the *A. nidulans* strains used in this study are shown in Table 1, the DNA repair proficient diploid strain UT448//A757 and the DNA repair deficient diploid strain B211//A837 being prepared according to the method of Roper (1952). Czapek-Dox medium supplemented with 1% (w/v) glucose was used as the Minimal Medium (MM) while the Complete Medium (CM) was that described by Pontecorvo *et al*. (1953) and Van de Vate and Jansen (1978). Supplemented medium (SM) consisted of MM plus the nutrients required by each strain. Solid medium contained 1.5% (w/v) agar.

Evaluation of sodium nitroprusside toxicity

Conidia of *A. nidulans* haploid strain A757, derived from colonies grown in CM, were inoculated at the center of six CM plates (the control group) and six plates containing CM supplemented with various concentrations (40, 80, 160 or 320 μM) of sodium nitroprusside (CM+SN) (the experimental group). The plates were incubated at 37 °C and the diameter of the colonies measured after 24, 48, 72, 96 and 120 h incubation. The growth rates of the control and experimental groups were compared using the Student's *t* test at $p < 0.05$.

Table 1 - Genotype and origin of *Aspergillus nidulans* strains.

Strains	Genotype[$]	Origin[#]
UT448	*riboA1, pabaA124, biA1* (I); *AcrA1, wA2* (II)	Utrecht Stocks
B211	*yA2; biA1; AcrA1; wA2; methA17; uvsH77, pyroA4, chaA1*	LGM
A837	*pabaA1, uvsH77, pyroA4, choA1, chaA1*	FGSC
A757	*yA2, methA17, pyroA4*	FGSC

[$]Mutant allele phenotypes. Requirement for: riboflavin = *riboA1*; *p*-aminobenzoic acid = *pabaA124* or *pabaA1*; biotin = *biA1*; methionine = *methA17*; pyridoxine = *pyroA4*; and choline = *choA1*. Conidia color: chartreuse = *cha*; *w* = white; and *y* = yellow. Miscellaneous: Acriflavine resistance = *AcrA1*; sensitivity to UV light = *uvsH77*.
[#]FGCS = Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas USA; LGM = Laboratory of Genetics of Microrganisms, State University of Maringá, Maringá, Paraná, Brazil.

Spore production

Conidia from strain A757, derived from colonies grown in CM, were inoculated at the center of six plates containing CM (the control group) and six containing CM+SN (the experimental group) (40 to 320 μM) which were incubated for 24 h at 37 °C. After incubation spore production was determined by washing each plate with 15 mL of 0.01% (w/v) Tween 80 and 20 sterilized glass beads (3 mm ϕ) to produce a suspension of conidia which were counted using a haemacytometer. Results for the control and experimental groups were compared using the Student's *t* test at $p < 0.05$.

Calculating homozygotization index

Conidia of the diploid *A. nidulans* strains (UT448//A757 and B211//A837) were individually inoculated onto MM+SN plates containing different concentrations of sodium nitroprusside (40, 80 or 160 μM) and incubated for 5 days at 37 °C. This treatment produced 19 visible mitotic morphologically identifiable diploid sectors (D1 to D19) which differed from the original diploid strain. The sectors were homozygous (+/+) or heterozygous (+/- or -/+) diploids but never recessive homozygotes (-/-) because these cannot grow on MM. The new diploid strains (D1 to D19) were purified on MM, individually transferred to CM plates and processed by spontaneous haploidization. After haploidization, the haploid mitotic segregants, obtained from diploids D1-D18, were purified in CM and their mitotic stability evaluated. Only mitotically stable segregants at the final stage were selected for the recombinagenesis test (Chiuchetta and Castro-Prado, 2002).

For the recombinagenesis tests conidia of each haploid segregant were individually transferred to 25 positions on CM plates and incubated for 48 h, after which colonies were transferred to different supplemented media: MM supplemented with all of the nutritional requirements of the master strains (UT448 and UT196 or B211 and A837), being omitted one of them, in each type of medium. Mitotic crossing-over causes homozygotization of heterozygous-conditioned genes. If sodium nitroprusside induces mitotic crossing-over in diploid strains UT448//A757 and B211//A837 only heterozygotes (+/- or -/+) or homozygotes (+/+) diploids will develop in Minimal Medium and the nutritional markers will segregate among the haploids in the proportion of 4+ to 2- but if sodium nitroprusside does not induce crossing-over the proportion will be 4+ to 4- because the initial selection process limits the growth of -/- diploids. The ratio of prototrophic to auxotrophic segregants is described by the Homozygotization Index

(HI), a HI value equal to or higher than 2 indicating that sodium nitroprusside has recombinogenic effects (Pires and Zucchi, 1994). The recombinogenic potential of sodium nitroprusside was assessed by comparing the homozygotization indices of the *p*-aminobenzoic acid (*paba*), biotin (*bi*), methionine (*meth*) and pyridoxine (*pyro*) genes using the Chi-squared (χ^2) test with the Yates correction and $p < 0.05$.

Results and Discussion

We found that, as compared to controls which received no sodium nitroprusside, 40 to 320 μM of sodium nitroprusside modified the mycelial growth of strain A757 and reduced the conidia production (Table 2, Figure 1) but did not affect the temporal development of cells (data not shown), these results being in agreement with those of Ninnemann and Maier (1996) who demonstrated sodium nitroprusside inhibited conidiation in *Neurospora crassa.*

Conidiation in *A. nidulans* requires a transition from the polarized growth pattern of vegetative hyphae to apolar budding of uninuclear cells. The products of at least two major regulatory genes, *brlA* (bristle) and *stuA (*stunted), are transcription factor proteins that regulate the asexual sporulation in *A. nidulans* (Timberlake and Clutterbuck, 1994; Wu and Miller, 1997). The *brlA* gene encodes a C_2H_2 zinc finger protein mediating the transition from polarized to radial growth at the stalk apex of the asexual spore-forming conidiophore to form the conidiophore vesicle (Aguirre, 1993; Adams *et al*., 1988) while regulated *stuA* expression is required for correct cell-pattern formation during asexual reproduction (Wu and Miller, 1997). We suggest that alterations in *A. nidulans* conidiation induced by sodium nitroprusside may be due to nitric oxide impairing the transcriptional activation of structural sporulation-specific genes. It has already been shown that nitric oxide inhibits both the DNA binding activity of yeast zinc finger LAC9 and non-zinc finger CPF-1 transcription factors (Kröncke, 2001; Berendji *et al*., 1999; Kröncke *et al*., 1994).

Table 2 - Influence of 40 to 320 μM of sodium nitroprusside on conidiation in *Aspergillus nidulans* strain A757.

	Sodium nitroprusside concentration (μM)				
	0[#]	40	80	160	320
Conidia per mL x 10^3	720.6	235.6*	149.4*	8.1*	7.0*

[#]Control.
*Significantly different from control (Student's *t* test, $p < 0.05$). The arithmetic mean of four experiments was estimated for each treatment.

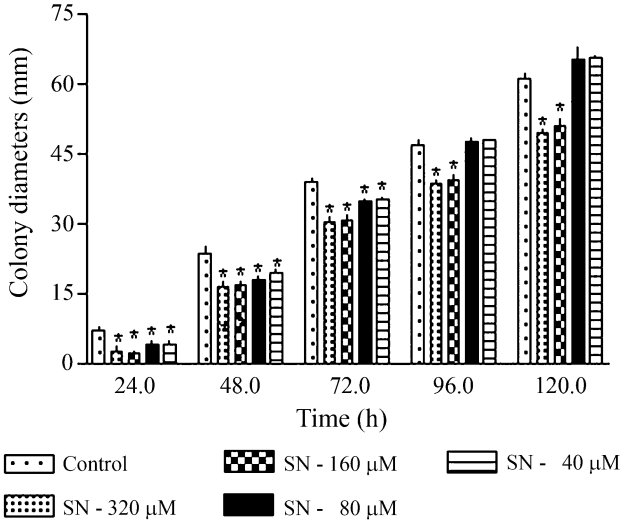

Figure 1 - Growth of *A. nidulans* strain A757 on plates containing Complete Medium plus various concentrations (0 (control), 40, 80, 160 and 320 μM) of sodium nitroprusside. An asterisk (*) indicates that growth was significantly different from the control (*t* test, $p < 0.05$).

We evaluated the recombinogenic potential of sodium nitroprusside towards *A. nidulans* diploid strains UT448//A757 (repair proficient) and A837//B211 (repair deficient) using the homozygotization index of various nutritional markers, homozygotization indices for the *bi*, *paba* and *pyro* genes being significantly higher in diploid strains treated with sodium nitroprusside (strains D2 to D4 and D6 to D18) as compared to untreated control strains (Tables 3 and 4). Diploid strains D1 and D5, derived from strain UT448//A757 treated with sodium nitroprusside, showed homozygotization indices of greater than 2 for the *paba* and *bi* genes but these results were not statistically significant (Table 3). In contrast, however, all the diploid strains obtained after sodium nitroprusside treatment of strain A837//B211 (strains D10 to D18) showed *bi*, *paba* and *pyro* genes homozygotization indices higher than 2 and significantly different from control values (Table 4). Kafer and Mayor (1986) and Yoon *et al.* (1995) showed that post-replication repair deficient *A. nidulans uvsH* mutants such as strains A837 and B211 have a high frequency of spontaneous mitotic recombination in homozygous condition, a fact which explains the higher sodium nitroprusside sensitivity of the repair deficient diploid strain A837//B211 than the repair proficient strain UT448//A757.

We obtained *meth* gene homozygotization indices of less than 2 for A837//B211 strains D10 to D18 (Table 4) and this may have been due to the greater number of white (*w*) segregants isolated after haploidization, because in *A. nidulans* strain B211 the *w* gene is closely linked to the *meth* gene and consequently most of the *w* segregants have the *meth* phenotype.

Although auxotrophic diploids (-/-) are not selected for in Minimal Media supplemented with sodium nitroprusside, recessive homozygous diploids may be obtained for coloration markers of conidia such as the *w* gene. In our experiments only prototrophic diploids with chartreuse conidia (cha//*cha*) were isolated from diploid B211//A837

Table 3 - Total number of segregants (NS) and homozygotization Indexes (HI) for markers belonging to *Aspergillus nidulans* DNA repair proficient diploid strain UT448//A757 after treatment with 40 μM (D1-D3), 80 μM (D4-D6) or 160 μM (D7-D9) of sodium nitroprusside.

Strain[#]	Control[$]		D1		D2		D3		D4		D5		D6		D7		D8		D9	
	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI
bi+	169	1.41	44	2.00	42	2.63	46	2.88*	45	3.75*	35	2.33	49	3.77*	51	3.64*	40	3.08*	32	1.03
bi	120		22		16		16		12		15		13		14		13		31	
paba+	173	1.49	46	2.30	44	3.14*	43	2.26	41	2.56	36	2.57	48	3.43*	53	4.42*	42	3.82*	51	4.25*
paba	116		20		14		19		16		14		14		12		11		12	
pyro+	150	1.08	35	1.59	32	1.07	29	1.38	35	1.59	29	1.38	32	1.07	42	1.27	30	1.30	35	1.25
pyro	139		22		30		21		22		21		30		33		23		28	

[#]*bi* = biotin; *paba*= *p*-aminobenzoic acid; and *pyro* = pyridoxine.
[$]Not treated with sodium nitroprusside.
*significantly different from control ($p < 0.05$).

Table 4 - Total number of segregants (NS) and homozygotization Indexes (HI) for markers belonging to *Aspergillus nidulans* DNA repair deficient diploid strain A837//B211 after treatment with 40 μM (D10-D12), 80 μM (D13-D15) or 160 μM (D16-D18) of sodium nitroprusside.

Strain[$]	Control[#]		D10		D11		D12		D13		D14		D15		D16		D17		D18	
	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI	NS	HI
paba+	73	1.97	55	4.58*	37	2.46	54	4.50*	31	1.72	45	5.00*	40	5.71*	41	5.13*	34	3.78	39	5.57*
paba	37		12		15		12		18		9		7		8		9		7	
bi+	70	1.75	50	2.94	42	4.20*	36	1.20	40	4.44*	44	4.40*	33	2.35	29	1.45	36	5.14*	38	4.75*
bi	40		17		10		30		9		10		14		20		7		8	
meth+	11	0.11	11	0.19	5	0.11	7	0.12	10	0.26	9	0.16	9	0.24	6	0.14	5	0.13	8	0.21
meth	99		56		47		59		39		55		38		43		38		38	

[$]*bi* = biotin; *meth*= methionine; and *paba*= *p*-aminobenzoic acid.
[#]Not treated with sodium nitroprusside.
*significantly different from control ($p < 0.05$).

treated with sodium nitroprusside, although prototrophic diploids with green (*y+//y*) and white (*w//w*) conidia were isolated from colonies of diploid UT448//A757 treated with sodium nitroprusside. Phenotypic analysis showed diploid D19 (white) to be a recombinant for the centromere-*meth* interval of chromosome II (Figure 2).

The recombinogenic effect of sodium nitroprusside in diploid *A. nidulans* cells may reflect a direct genotoxic effect of nitric oxide on DNA, stimulating the occurrence of DNA breaks during the G2 period. It is also possible that the same genotoxicity effect operates in mammalian cells and may contribute towards the nitric oxide tumor promoting effect.

The transformation of normal human cells into cancer cells is a multistep process and mitotic recombination is a factor that may be involved in the overall transformation process. Since mitotic crossing-over promotes homozygotization of recessive genes, studies have been developed to identify possible recombinogenic agents. Our results show that at concentrations between 40 μM to 160 μM the nitric oxide donor sodium nitroprusside is efficient at inducing mitotic crossing-over in diploid strains of *A. nidulans*.

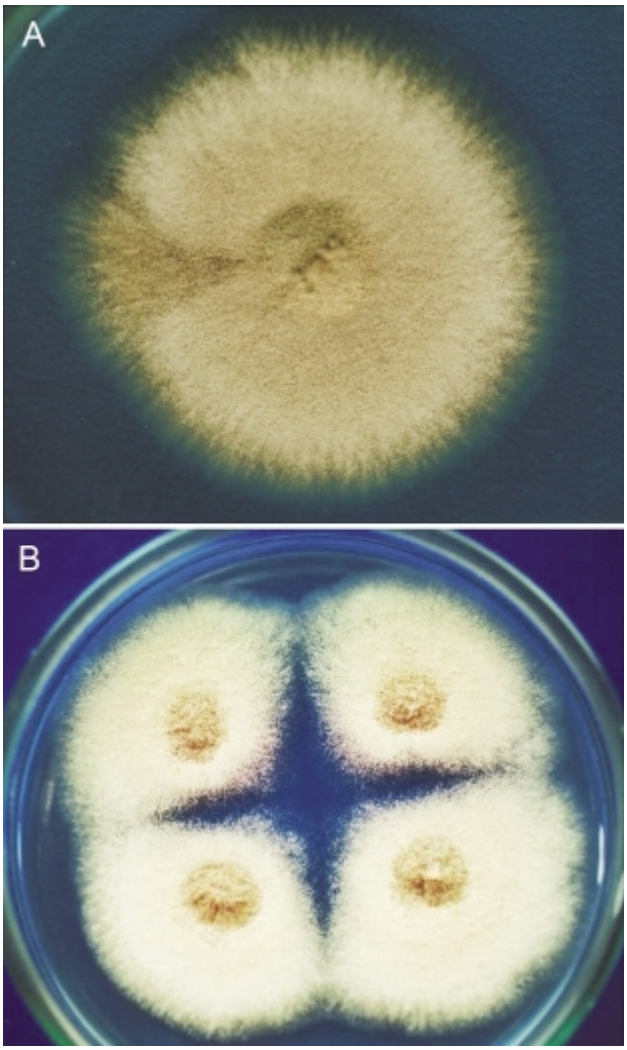

Figure 2 - (A) Mitotic instability of diploid strain D19 obtained after sodium nitroprusside treatment (160 μM) of diploid strain UT448//A757; (B) stable mitotic segregant derived from strain D19.

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Associate Editor: Sérgio Olavo Pinto da Costa