Participation of nitric oxide in the color change induced by UV radiation in the crab Chasmagnathus granulatus

Article *in* Pigment Cell & Melanoma Research · May 2008

DOI: 10.1111/j.1755-148X.2007.00439.x · Source: PubMed

CITATIONS
16

READS
122

9 authors, including:

Bruno Cruz
Universidade Federal do Rio Grande (FURG)
12 PUBLICATIONS **193** CITATIONS
SEE PROFILE

Fábio Everton Maciel
Universidade Federal do Rio Grande (FURG)
28 PUBLICATIONS **471** CITATIONS
SEE PROFILE

Márcio Alberto Geihs
Universidade Federal do Rio Grande (FURG)
25 PUBLICATIONS **413** CITATIONS
SEE PROFILE

Ana Luiza Muccillo-Baisch
Universidade Federal do Rio Grande (FURG)
103 PUBLICATIONS **1,059** CITATIONS
SEE PROFILE

Participation of nitric oxide in the color change induced by UV radiation in the crab *Chasmagnathus granulatus*

Marcelo Alves Vargas[1], Bruno Pinto Cruz[1], Fábio Everton Maciel[1], Marcio Alberto Geihs[1], João Carlos Brahn Cousin[2], Gilma Santos Trindade[1], Ana Luiza Muccillo Baisch[1], Silvana Allodi[3*†] and Luiz Eduardo Maia Nery[1†]

[1]Fundação Universidade Federal do Rio Grande – FURG, Departamento de Ciências Fisiológicas, Programa de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Rio Grande, RS, Brazil
[2]Fundação Universidade Federal do Rio Grande – FURG, Departamento de Ciências Morfobiológicas, Programa de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Rio Grande, RS, Brazil
[3]Universidade Federal do Rio de Janeiro, Departamento de Histologia e Embriologia, Instituto de Ciências da Biomédicas, 21949-900, Rio de Janeiro, RJ, Brazil
*Address correspondence to Silvana Allodi, e-mail: sallodi@histo.ufrj.br
[†]S. Allodi and L. E. M. Nery contributed equally to the paper.

Summary

The ability of UV radiation to stimulate color change in vertebrates is well known; however, the signaling pathway involved is not fully explained. Since nitric oxide (NO) is among the candidates for this role, in this study the participation of NO signaling in the pigment migration induced by UV radiation in melanophores of the crab *Chasmagnathus granulatus* was investigated. When the NO donor, SIN-1, was incubated with pieces of epidermis, there was an induction of a dose-dependent pigment dispersion (in vitro assays). When male adults were exposed to different doses of UVA and UVB, N^G-nitro-L-arginine-methyl-ester, an NO synthase (NOS) blocker produced a decrease of the pigment dispersion induced by UV (in vivo assays). However, in similar assays, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, an NO scavenger, decreased only the pigment dispersion induced by UVA. Interestingly, buthionine sulfoximine did not produce any change in pigment dispersion induced by UVA (in vivo assays) and SIN-1 (in vitro assays). Our results using NADPH-diaphorase histochemistry and immunocytochemistry against nNOS indicated the production of NO by epidermal cells. In conclusion, we suggest that NO is a key molecule for the induction of pigment dispersion in the melanophores of *Chasmagnthus granulatus*, and also that NOS activation is a fundamental step for this process.

Key words: nitric oxide/UV radiation/melanophores/pigment dispersion/*Chasmagnathus granulatus*

Received 22 March 2006, revised and accepted for publication 1 November 2007

doi: 10.1111/j.1755-148X.2007.00439.x

Introduction

Many environmental factors are determinant to induce color change, such as background color, photoperiod, and temperature. Another important factor that induces color change is solar radiation, including visible and ultraviolet radiation (UV) (Oshima, 2001). UV radiation comprises an electromagnetic wavelength between 200 and 400 nm. In order to analyze the ability to cause damage in cellular targets, UV was divided into UVA (320–400 nm), UVB (290–320 nm) and UVC (200–290 nm). The wavelength of UV that includes UVA and UVB is also called Solar UV (Diffey, 2002). The ability of UV radiation to stimulate color change in vertebrates is well known (Allan et al., 1995; Bagnara and Hadley, 1973; Gilcherest et al., 1999; Lesser, 2001); however, the signaling pathway of UV radiation remains to be fully elucidated. Two means of stimulation are suggested for this process: the direct action of UV on the pigment cells, or indirect action with production of some paracrine agonists for other non-pigmented cells. Among the paracrine factors involved in the exposure of UV are endothelins, eicosanoids, POMC hormones such as α-MSH and ACTH, and histamines. Furthermore, nitric oxide (NO) has received special attention as a paracrine factor associated with the UV-induced response (Romero-Graillet et al., 1996).

NO is one of the smallest and simplest molecules that have been biosynthesized. It is a gaseous molecule, formed by one atom of nitrogen (N) and one atom of oxygen (O), found naturally in the atmosphere in small quantities; it is very toxic because of the presence of

free radicals. The substrate for synthesizing O in the organism is L-arginine. In addition to participating in the urea cycle, L-arginine is involved in the synthesis of creatine and polyamides (Konturek and Konturek, 1995). The enzymes that convert L-arginine to L-citruline and NO are called nitric-oxide synthases (NOS). These enzymes are hemeproteins of the 'cytochrome-like family'. For NOS to produce NO, they depend on O_2, NADPH, flavines and biopterines.

Up to the present, three isoforms of the NOS have been isolated and cloned (Bredt and Snyder, 1990; Lyons et al., 1992; Nishida et al., 1992): two constitutive and one inducible. The cNOS (constitutive nitric oxide synthesis), which include the eNOS (endothelial nitric oxide synthesis) and the nNOS (neuronal nitric oxide synthesis), differ from the iNOS (inducible nitric oxide synthesis) in their molecular weight, their form of activation and their ability to synthesize NO (Marletta, 1994).

NO is a highly diffusible and reactive molecule. Therefore, instead of activating downstream pathways by traditional receptor-mediated events, it induces its biological effects by directly activating many intercellular molecules. Wink and Mitchell (1998) classified these NO reactions as being either direct (on the biological mediator) or indirect (involving reactive nitrogen and oxygen species). The direct downstream pathways consist mainly of interactions between NO and heme-containing proteins, the most important being guanylate cyclase (McDonald and Murad, 1996 and Murad, 1994). Activation of this enzyme by NO induces the production of cyclic guanosine 3,5-monophosphate (cGMP), which in turn activates protein kinase G (PKG). This downstream pathway is particularly important in mediating the effects of the low levels of NO production, which seems to occur with cNOS activation. The indirect downstream pathways, which become more important under high local concentrations of NO, involve the formation of nitrogen oxide species such as N_2O_3, HNO (nitroxyl), and $ONOO^-$ (peroxynitrite) (Feelisch et al., 2002). These molecules, in turn, modify thiol-containing proteins, either by nitrosation (N_2O_3) or oxidation (HNO and $ONOO^-$). The selection of the indirect downstream pathway seems to depend, to some extent, on the redox potential of the cell (Lipton et al., 1993; Miranda et al., 2003). UV irradiation also directly activates certain transmembrane receptors, such as the epidermal growth factor (EGF) receptor (Miller et al., 1994) and the keratinocyte growth factor (KGF) receptor (Marchese et al., 2003), which continue their action activating downstream pathways and initiating the production of peroxide and reactive oxygen species (ROS).

The evidence of a central role for NO in the induction of melanogenesis has been apparent since the mid-1990s, when Romero-Graillet et al. (1996, 1997) found that UV-irradiated human melanocyte cultures produced NO and that the presence of NO was sufficient to induce melanogenesis. Hayashi and Fujii (2001) reported that NO donors also induce the pigment dispersion in melanophores of teleosts. In other systems, it is known that in insects NO is involved in olfactory signaling (Sapura et al., 2006) and in crustaceans, the expression of NOS has been established in the nerve gland, Y-organ, gill and gonad (Kim et al., 2004). Furthermore, recent studies have shown that the NO pathway is involved in neuronal development and neuron, skeletal muscle and cardiac muscle regulation in crustaceans (Aonuma et al., 2000; Aonuma and Newland, 2001, 2002; Erxleben and Hermann, 2001; Hermann and Erxleben, 2001; Johansson and Mellon, 1998; Mahadevan et al., 2004) and regulation of molting (Lachaise et al., 1993; Lee and Mybles, 2006; Skinner, 1985), but its participation in crustacean color-change regulation is unknown.

In crustaceans, color change is principally associated with the X-organ/sinus gland neuroendocrine complex. To date, only two hormones affecting the color change, released mainly from the X-organ/sinus gland neuroendocrine complex, are well known: the pigment-dispersing hormone (PDH) and the red pigment-concentrating hormone (RPCH).

The crab *Chasmagnathus granulatus* is a semi-terrestrial animal of the southern coast of Brazil, Uruguay and Argentina. A former study showed that pigment dispersion occurs in this crab's melanophores during UV exposure (Gouveia et al., 2004). This dispersion rapidly disappears when the stimuli cease, returning to the aggregated stage, 45 min after the exposure. Interestingly, this color change occurs also in eyestalkless crabs; therefore, it is independent from the X-organ/sinus gland complex and its neurohormones. In this study, we wanted to investigate whether NO participates in the color-change signaling induced by UVA and UVB in melanophores of the crab *C. granulatus*.

Results

The melanophores of *C. granulatus* showed significant ($P < 0.05$) dose-dependent pigment dispersion when incubated with a potent NO donor, SIN-1. The minimal effective dose of SIN-1 that induced pigment dispersion was 10^{-8} M, and maximum pigment dispersion was obtained with 10^{-5} M. The EC_{50} (with 95% confidence interval) was 1.2 (0.3–4.2) $\times 10^{-6}$ M (Figure 1A).

In in vivo assays, UVA exposure induced significant dose-dependent pigment dispersion in *C. granulatus* melanophores. However, when the crabs were treated with N^G-nitro-L-arginine-methyl-ester (L-NAME), they showed an inhibition of pigment dispersion compared with the control group ($P < 0.05$) (Figure 1B). The UVB exposure also induced significant dose-dependent pigment dispersion. Similarly, the crabs treated with the blocker L-NAME also showed less pigment dispersion

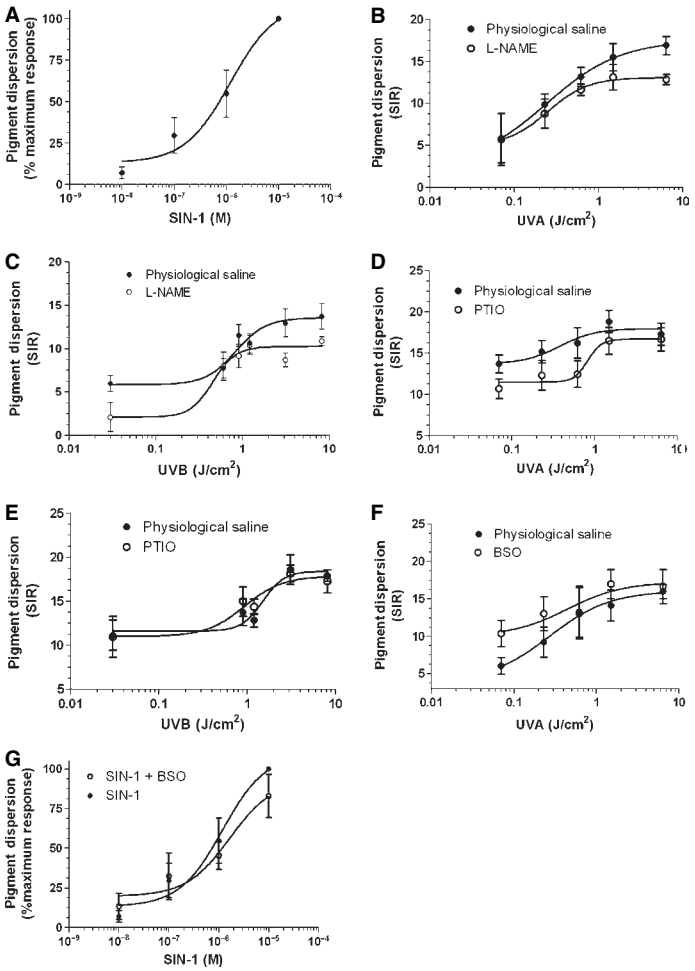

Figure 1. Dose–response curves of modulators of NO from UV exposed (in vivo) and non-exposed (in vitro) *Chasmagnathus granulatus*. (A) In vitro dose–response curve for SIN-1 of melanophores. Each point represents the mean (±SEM) of the dispersing response at the concentration noted (n = 5). (B) In vivo dose–response curve for UVA-induced pigment dispersion in crabs previously injected with L-NAME and physiological saline. Each point represents the mean (±SEM) of the dispersing response, measured as standard integrated response (SIR) at the UVA dose noted (n = 5). (C) In vivo dose–response curve for UVB-induced pigment dispersion in crabs previously injected with L-NAME and physiological saline. Each point represents the mean (±SEM) of the dispersing response, measured as standard integrated response (SIR) at the UVB dose noted (n = 8). (D) In vivo dose–response curve for UVA-induced pigment dispersion in crabs previously injected with PTIO and physiological saline. Each point represents the mean (±SEM) of the dispersing response, measured as standard integrated response (SIR) at the UVA dose noted (n = 4). (E) In vivo dose–response curve for UVB-induced pigment dispersion in crabs previously injected with PTIO and physiological saline. Each point represents the mean (±SEM) of the dispersing response, measured as standard integrated response (SIR) at the UVB dose noted (n = 5). (F) In vivo dose–response curve for UVA-induced pigment dispersion in crabs previously injected with BSO and physiological saline. Each point represents the mean (±SEM) of the dispersing response, measured as standard integrated response (SIR) at the UVA dose noted (n = 4). (G) In vitro dose–response curve for SIN-1 with BSO of melanophores. Each point represents the mean (±SEM) of the dispersing response at the concentration noted (n = 5).

compared with the control group ($P < 0.01$) (Figure 1C). UVA radiation was more effective than UVB radiation in inducing pigment dispersion (Figure 1B,C). Another notable point is that L-NAME does not induce any pigment dispersion in non-irradiated crabs.

2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) produced different effects on animals exposed to UVA and UVB. When exposed to UVA, the animals treated with PTIO responded with inhibition of pigment dispersion, different from those that received physiological saline ($P < 0.05$) (Figure 1D). In contrast, in the animals exposed to UVB there was no difference in the pigment dispersion response between PTIO and control groups ($P > 0.05$) (Figure 1E). Also, it is important to note that PTIO does not induce pigment dispersion in non-irradiated crabs. In addition, buthionine sulfoximine (BSO) did not result in significant ($P > 0.05$) change in UVA- or SIN-1-induced pigment dispersion (Figure 1F,G, respectively). BSO also did not induce pigment dispersion in non-irradiated cabs.

Figure 2 shows a eyestalkless crab before (A) and after (B) UV exposure. Observe that the irradiated crab displays an expressive pigment dispersion (Figure 2B).

A layer of basically two cell types constitutes the epidermis of the crab *C. granulatus*, below the exoskeleton: the epithelial cells and the chromatophores: xanthophores, erythrophores and melanophores (Figure 3A). The epidermis of the pereiopods of *C. granulatus* not exposed to UV showed a positive reaction for both techniques employed (NADPH-diaphorase histochemistry and nNOS immunohistochemistry). Compared with the control (Figure 3B), the NADPH-diaphorase histochemical analysis showed a positive reaction over the entire epidermis of the crab, revealing the production of NO in most cells of this tissue (Figure 3C). Confirming this result, the immunohistochemical reaction against nNOS labeled the epidermis (Figure 3D). After the exposure of the crabs to UV, both the NADPH-diaphorase histochemistry and the nNOS immunohistochemistry showed stronger reactions than those using non-exposed crabs (Figure 3E,F, respectively).

Discussion

Several studies have demonstrated a relationship between increased pigmentation in mammals and UV radiation (Gilcherest et al., 1999; Hill and Hill, 2000; Murphy et al., 2001). In addition, some studies have also reported that the tanning effect induced by UV radiation occurs in other vertebrate groups. Juvenile hammerhead sharks showed an increase in the skin melanin content in direct response to UV (Lowe and Goodman-Lowe, 1966), and spotted salamander embryos also increased melanin production in response to UV radiation (Lesser, 2001). This effect has also been observed in pigment dispersion. In crustaceans, melanophores of the fiddler crab *Uca pugilator* (Coohill et al., 1970) and *C. granulatus* (Gouveia et al., 2004) showed immediate pigment dispersion in response to UV radiation. However, besides causing an increase in melanin and pigment dispersion, most of the signaling mechanisms triggered by UV radiation are still unknown.

As far as we know, in invertebrates, the participation of NO in the pigment dispersion induced by UV irradiation was never addressed. Therefore, we wanted to verify whether this phenomenon also occurs in crustaceans. The results of the present study show that the production of NO can induce pigment dispersion in melanophores of *C. granulatus*. These cells show a dose-dependent pigment dispersion after treatment with SIN-1, a potent NO donor. In vertebrates, Hayashi and Fujii (2001) also observed that the increase in NO production induces pigment migration in melanophores of two teleost species treated with various NO donors. In contrast, Oberg et al. (1999) reported that NO production in melanophores of an amphibian species is required for aggregation of melanosomes.

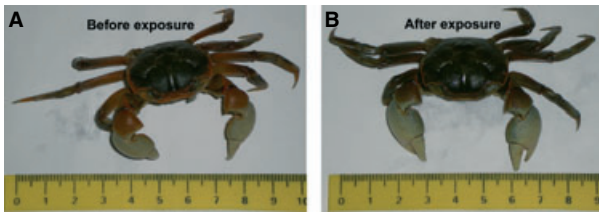

Figure 2. *Chasmagnathus granulatus* before (A) and after (B) exposure to UV radiation.

Regarding melanogenesis, the effects of NO are similar to the effects of NO in pigment dispersion. It was observed that human melanocytes markedly increase tyrosinase activity and melanin neosynthesis in a dose-dependent manner when treated with NO donors (Romero-Graillet et al., 1996). Furthermore, the NO donors also enhanced the melanocyte dendrocytes, indicating an increase in melanocyte activity. The NO-induced melanogenesis was accompanied by an upregulation of tyrosinase gene expression (Sasaki et al., 2000). NO also may be involved with the production and increased eumelanin/pheomelanin ratio (Lassalle et al., 2003). Theses results suggest that NO can induce color change in animal groups, being a chromatophoric agonist in various animals.

The next step in the present study was to investigate whether NO is involved with the pigment dispersion induced by UV in *C. granulatus*. Romero-Graillet et al. (1996) reported that human keratinocytes exposed to UVA and UVB showed increased NOS activity. Furthermore, when irradiated keratinocytes were co-cultured with non-irradiated melanocytes, they induced an increase in tyrosinase activity, indicating that the amount of NO released to keratinocytes was sufficient to induce melanogenesis. In addition, when the same experiment was performed in the presence of two NO scavengers (hemoglobin and carboxy-PTIO), the increase of tyrosinase activity induced by keratinocytes was completely blocked. However, Romero-Graillet et al. (1997) also reported that melanogenesis increase in human melanocytes directly irradiated with UVB is dependent on NO production. This molecule can also act as an autocrine factor or as an intracellular messenger to induce melanogenesis. In our study, UVA-induced dose-dependent pigment dispersion in melanophores of *C. granulatus*, and when a treatment with L-NAME (NOS blocker) or PTIO (NO scavenger) was performed, less pigment dispersion was observed. The results indicate that NOS activation and NO production are important steps in UVA-induced pigment dispersion. We cannot exclude that extra-ocular chromatophorotropins might be induced by UV radiation and could stimulate either NO production or NO releasing. Therefore, UV radiation could stimulate these putative extra-ocular chromatophorotropins, which, by their turn, could stimulate pigment dispersion. On the other hand, our results

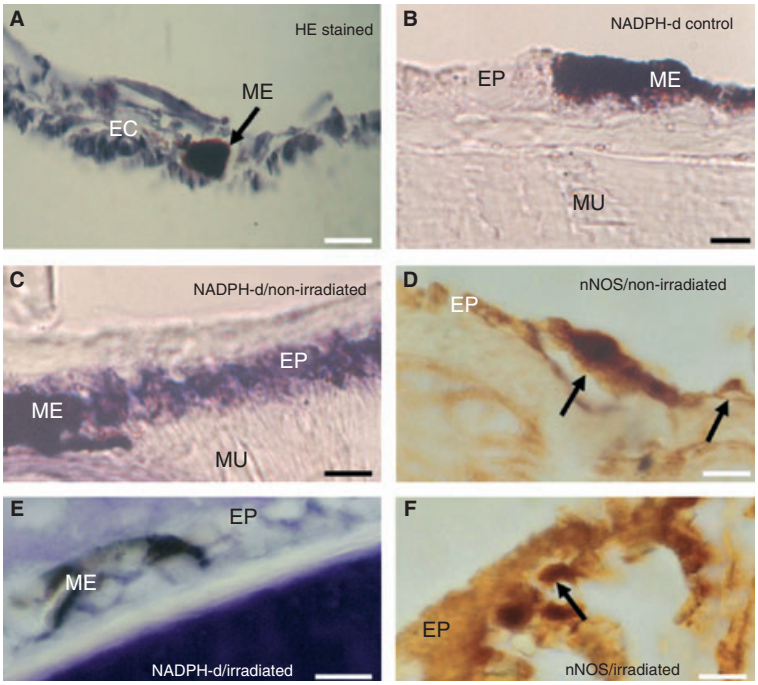

Figure 3. NOS location in the epidermis of the crab *Chasmagnathus granulatus*. (A) Epithelial cells (EC) and a melanophore (ME). Hematoxylin/eosin stained. (B) NADPH-diaphorase control reaction in the epidermis of the crab. (EP) epidermis, (ME) as in (A), (MU) muscle. (C) NADPH-diaphorase positive reaction in the epidermis of non-irradiated crabs. Note the reaction product in dark blue; Abbreviations are same as in (B). (D) Immunohistochemical reaction against nNOS. Observe the positive result (in light brown) in the cells of the epidermis (EP), including the melanophores (arrows), and in the muscle in non-irradiated crabs Abbreviations are same as in (A). (E) NADPH-diaphorase positive reaction in the epidermis (EP) of UV exposed crabs. (ME) as in (A). (F) Immunocytochemical reaction against nNOS in the cells of the epidermis (EP), including the melanophores (arrow), of crabs exposed to UV. Bars: 10 μm.

showed the importance of NO production on melanophore pigment dispersion induced by UV radiation.

NO production may also occur through other pathways besides NOS activation. In human skin, UVA irradiation leads to NO production due to photo-decomposition of nitroreactive species (NORS) and nitrite, representing the primary basis for NO formation during UVA exposure (Paunel et al., 2005). Thus, another point to verify was whether there is NO production by NORS photo-decomposition. For this purpose, we used an inhibitor of GSH production (BSO) that leads to an increase of NORS and consequently an increase of NO formation by photo-decomposition. Our results showed that BSO does not alter UVA and SIN-1-induced pigment dispersion in melanophores of *C. granulatus*. Therefore, the UVA-induced pigment dispersion is apparently dependent only on NOS activation. However, we cannot discard the possibility that the concentration of BSO used did not produce an effective increase of NORS, because the same concentration was able to increase NORS in rat hepatocytes (Yoshida et al., 2005).

L-NAME was also able to reduce the UVB-induced pigment dispersion in melanophores of *C. granulatus*. The L-NAME effect is similar to experiments with guinea pigs, in which topical application of L-NAME inhibited UVB-induced melanogenesis (Horikoshi et al., 2000). Furthermore, the increase in the number of DOPA-positive melanocytes and melanin content in the skin, essential for hyperpigmentation, was also reduced. However, PTIO did not alter the UVB-induced pigment dispersion in melanophores of *C. granulatus*. It is possible that the PTIO dose used was not sufficient to change significantly the UVB-induced NO production. It is also possible that the action mechanisms of the two types of UV radiation, UVA and UVB, are different. It is interesting to observe that there is a difference between the ability of UVA and UVB to induce color change in mammals and crustaceans. The response induced by UVA is two to three orders of magnitude less efficient than UVB in mammals (Eller and Gilchrest, 2000). In *C. granulatus*, UVB radiation is less effective than UVA radiation in inducing pigment dispersion (Gouveia et al., 2004, and present results).

Clearly, the results obtained in vitro lead to the conclusion that SIN-1 stimulated pigment dispersion. Similar results were obtained in vivo, when we used L-NAME and PTIO in UVA and UVB irradiated crabs. Surprisingly, however, PTIO only blocked pigment dispersion after UVA irradiation. In addition, in vitro and in vivo assays using BSO demonstrated that ROS production was not sufficient to produce expressive pigment dispersion. Therefore, we may suggest that both strategies were able to demonstrate the importance of NO in triggering pigment dispersion.

The last step was to verify the presence of NOS and its location in the epidermis of *C. granulatus*. In mammals, all skin cell types express NOS and seem capable of releasing NO. Although a few early reports suggested that human keratinocytes expressed eNOS (Sakai et al., 1996; Shimizu et al., 1997), further experiments suggested that perhaps a post-translational product of the NOS protein cross-reacted with certain anti-eNOS antibodies (Jackson et al., 1998). Most of the evidence now indicates that keratinocytes constitutively express nNOS (Sirsjo et al., 1996; Romero-Graillet et al., 1997). Further-

more, Jackson et al. (1998) found no induction of eNOS mRNA following the exposure of keratinocytes to UVB irradiation or IFN-γ. Keratinocytes are also known to express iNOS and protein following exposure to inflammatory cytokines (Sirsjo et al., 1996; Frank et al., 1998). Normal human melanocytes also have been shown to express eNOS mRNA (Jackson et al., 1998), although immunonoreactive iNOS was reported in one study (Ivanova et al., 1997). iNOS also seems to be expressed by some malignant melanomas (Ahmed and Van Den Oord, 1999). Normal human melanocytes express iNOS following incubation with LPS, TNF-α or INF-γ (Fecker et al., 2002; Rocha and Guillo, 2001). In our study, the epidermis of *C. granulatus* was NADPH-diaphorase positive and clearly expressed nNOS. Both techniques revealed the presence of NOS in the majority of epidermal cells. These results demonstrated for the first time the expression of NOS in the epidermis of crustaceans.

In conclusion, our results showed that NO is an important molecule to induce pigment dispersion in melanophores of *C. granulatus*. In addition, NOS seems to be associated with UV radiation signaling to induce color change.

Material and methods

Animal capture and acclimation

Adult male crabs *C. granulatus*, weighing 7.0 ± 0.5 g, were collected in salt marshes near the city of Rio Grande in southern Brazil. The crabs were kept in tanks with salt water at 20% salinity, 20°C and photoperiod 12:12 L:D, for at least 7 days before the assays. During this period, the crabs were regularly fed with ground beef.

Reagents and solutions

The modulators of NO used in this experiment were purchased from Sigma (St Louis, MO, USA) (L-NAME, PTIO and BSO) and from Cayman (Ann Arbor, MI, USA) (SIN-1). The stock solutions were made up in distilled water (L-NAME), ethanol (PTIO) or DMSO (SIN-1). For each solution, the final dilution was made in physiological saline, and the final concentration of the solvents never exceeded 1%. The solution of BSO was made up directly in physiological saline. The physiological saline composition was, in mmol l^{-1}: $MgCl_2$, 0.01; NaCl, 0.355; $CaCl_2$, 0.016; H_3BO_3, 0.005; $KHCO_3$, 0.010; $Na_3C_6H_5O_7$, 0.008; pH 7.6.

In vivo assays

In the in vivo assays, only eyestalkless crabs were used. The ablation was done 24 h before the beginning of the experiment. The crabs received the injection (100 μL) of L-NAME (an analog and competitor of L-arginine; 500 nmol/crab), PTIO (an NO scavenger; 10 nmol/crab) or BSO (an inhibitor of γ-glutamylcysteine; 14.5 nmol/crab) 15 min before the exposure. The control group received only physiological saline injection (100 μL). For the BSO experiments, the control group received physiological saline with 1% DMSO; for the PTIO experiments, the control group received physiological saline with 1% ethanol.

The animals were irradiated for 30 min with different doses of UVA (6.4; 1.5; 0.6; 0.2; 0.07 J/cm^2) and UVB (8.1; 3.1; 1.2; 0.9; 0.6; 0.03 J/cm^2). The UVA (VL: 115 L, 30 W) or UVB (VL: 115 C, 30 W; Vilber Lourmat, Marne Lavalee, France) lamps were monitored using a radiometer/photometer (model IL 1400A, International Light, Newburyport, MA, USA). The UVA lamp irradiation was 1.39 mW/cm^2 UVA, with contamination of 0.006 mW/cm^2 UVB and 928.0 nW/cm^2 visible light. The UVB lamp irradiation was 1.195 mW/cm^2 produced with contamination of 493 μW/cm^2 of UVA and 0.113 μW/cm^2 of visible light. Both lamps showed no contamination with UVC. During UV exposure, the crabs were maintained at the same temperature (20°C).

An extra control group was composed of crabs that were not irradiated with UV, but maintained under fluorescent lamps (Philips TLT 40 W/75, São Paulo, Brazil) irradiating 96.0 mW/cm^2 visible light. These crabs also received injections (100 μL) of reagents (L-NAME, PTIO, or BSO) or physiological saline.

Observation

Pigment dispersion was assessed using the Hogben and Slome (1931) index, which defines stage 1 as that with full pigment aggregation, stage 5 as that with full dispersion, and stages 2, 3, and 4 being intermediate conditions. The region chosen for observation was the meropodite of the third pair of maxillipeds, because this region has a thin, light-colored exoskeleton, thus facilitating observation.

The degree of pigment dispersion was evaluated before injection, 15 min after injection and 0, 15, 30, 60, 90, 120, 150 min after UV exposure. From these results, the standard integrated response (SIR), as described by Fingerman and Yamamoto (1967), was calculated in order to determine dose–response curves (DRCs).

In vitro assays

In the in vitro assays, the meropodite of the third pair of maxillipeds of non-irradiated crabs was incubated in physiological saline for 30 min before the experiments. The pieces were then taped to a glass cover slip, which was turned upside down and mounted in a perfusion chamber, as described by Britto et al. (1990). This preparation received increasing concentrations of SIN-1 (0.01, 0.1, 1, and 10 μM). To test the effect of BSO, the pieces were previously incubated in BSO (300 μM) for 30 min before the experiments. Increasing concentrations of SIN-1 (0.01, 0.1, 1, and 10 μM) with BSO (300 μM) were placed in the perfusion chamber. For both tests, each concentration remained for 20 min in the chamber. The cumulative DRCs to SIN-1 were determined as follows: the physiological saline was replaced with NO donor concentration, and the dispersion response was evaluated under the light with the aid of an ocular micrometer. After the maximal response to a concentration was determined, the response was calculated as the percentage change in the apparent length of a previously selected melanophore process.

Histological analyses

NADPH-diaphorase histochemistry

Periopods of adult crabs were dissected and fixed in 4% paraformaldehyde for 4 h in 0.1 M phosphate-buffered saline (PBS), washed in PBS (three times) and immersed in PBS and sucrose (30%) overnight. The next day, the material was embedded in OCT (Miles Inc., Elkhart, IN, USA) and 16 μm sections were obtained using a cryostat. The histological sections were washed in PBS with Tween 20 (0.05%) for 5 min and incubated with a 100 μL substrate solution containing 1 mg/mL βNADPH (Sigma) and 1 mg/mL NBT (nitro-blue tetrazolium, Sigma) for 2 h at 37°C in a humid chamber. The sections were then washed in 0.05 M Tris/HCl pH 7.6, dehydrated in alcohol and mounted in Entellan (Merck).

Immunohistochemistry

The pereopods of adult crabs were fixed with Lillie solution for 30 days, embedded in paraffin and sectioned using a rotary microtome. Sections (10 μM thick) mounted on gelatin-coated slides were deparaffinized and washed in 0.1 M PBS for 5 min. The following procedure was performed using the Pro TaqsABC-POD kit (BIOCYC Gesellschaft für Biotechnologie und Recyclingverfahren mbH & Co. Entwicklungs KG, Luckenwalde, Brandenburg, Germany). To block the endogenous peroxidase, the sections were incubated with 3% H_2O_2 and washed in PBS. Blocking of unspecific binding was performed with the blocking reagent. Next, the slides were washed in PBS and incubated with the primary antibody (rabbit anti-nNOS; Sigma) overnight at 4°C. The next day, the sections were washed in PBS, incubated with the secondary antibody and washed again in PBS. The sections were then covered with streptavidin complex and incubated for 30 min. After they were washed in PBS, the sections were covered with fresh DAB chromogen solution and incubated until color intensity was satisfactory. The sections were then washed in distilled water and counterstained with hematoxylin, and the slides were mounted with Proataqs® (PARAmount).

Statistical analyses

The dose–response curve was obtained by using a non-linear regression model. To verify differences in pigment dispersion as a function of UV radiation or NO exposure, data were subjected to two-way analyses of variance. The significance level adopted was 95%.

Acknowledgements

This study was supported by FAPERGS. BPC holds a fellowship from FAPERGS, MAG holds a fellowship from CNPq, and FEM holds a fellowship from CAPES. We are grateful to Jorge Luis da Silva for technical assistance.

References

Ahmed, B., and Van Den Oord, J.J. (1999). Expression of the neuronal isoform of nitric oxide synthase (nNOS) and its inhibitor, protein inhibitor of nNOS, in pigment cell lesions of the skin. Br. J. Dermatol. *141*, 12–19.

Allan, A.E., Archambaut, M., Messana, E., and Gilchrest, B.A. (1995). Topically applied diacylglycerols increase pigmentation in guinea pig skin. J. Invest. Dermatol. *105*, 687–692.

Aonuma, H., and Newland, P.L. (2001). Opposing actions of nitric oxide on synaptic inputs of identified interneurones in the central nervous system of the crayfish. J. Exp. Biol. *204*, 1319–1332.

Aonuma, H., and Newland, P.L. (2002). Synaptic inputs onto spiking local interneurons in crayfish are depressed by nitric oxide. J. Neurobiol. *52*, 144–155.

Aonuma, H., Nagayama, T., and Takahata, M. (2000). Modulatory effects of nitric oxide on synaptic depression in the crayfish neuromuscular system. J. Exp. Biol. *203*, 3595–3602.

Bagnara, J.T., and Hadley, M.E. (1973). Chromatophores and Color Change: The Comparative Physiology of Animal Pigmentation (New Jersey: Prentice Hall).

Bredt, D.S., and Snyder, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A. *87*, 682–685.

Britto, A.L.M., Castrucci, A.M.L., Visconti, M.A., and Josefsson, L. (1990). Quantitative in vitro assay for crustacean chromatophorotropins and other pigment cell agonist. Pigment Cell Res. *3*, 28–32.

Coohill, T.P., Bartell, C.K., and Fingerman, M. (1970). Relative effectiveness of ultraviolet and visible light in eliciting pigment dispersion in melanophores of the fiddler crab. Uca pugilator. Physiol. Zool. *43*, 232–239.

Diffey, B.L. (2002). What is the light. Photodermatol. Photoimmunol. Photomed. *18*, 68–74.

Eller, M.S., and Gilchrest, B.A. (2000). Tanning as part of the eukaryotic SOS response. Pigment Cell Res. *13*(Suppl. 8), 94–97.

Erxleben, C., and Hermann, A. (2001). Nitric oxide augments voltage-activated calcium currents of Crustacea (*Idotea baltica*) skeletal muscle. Neurosci. Lett. *300*, 133–136.

Fecker, L.F., Eberle, J., Orfanos, C.E., and Geilen, C.C. (2002). Inducible nitric oxide synthase is expressed in normal human melanocytes but not in melanoma cells in response to tumor necrosis factor-alpha, interferon-gamma, and lipopolysaccharide. J. Invest. Dermatol. *118*, 1019–1025.

Feelisch, M., Rassaf, T., Mnaimneh, S., Singh, N., Bryan, N.S., Jourd'Heuil, D., and Kelm, M. (2002). Concomitant *S*-, *N*-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. FASEB J. *16*, 1775–1785.

Fingerman, M., and Yamamoto, Y. (1967). Daily rhythm of melanophore pigment migration in eyestalkless fiddler crabs, *Uca pugilator*. Crustaceana *12*, 303–319.

Frank, S., Madlener, M., Pfeilschifter, J., and Werner, S. (1998). Induction of inducible nitric oxide synthase and its corresponding tetrahydrobiopterin-cofactor-synthesizing enzyme GTP-cyclohydrolase I during cutaneous wound repair. J. Invest. Dermatol. *111*, 1058–1064.

Gilcherest, B.A., Mark, S.E., Geller, A.C., and Yaar, M. (1999). The pathogenesis of melanoma induced by ultraviolet radiation. New Engl. J. Med. *29*, 1341–1348.

Gouveia, G.R., Lopes, T.M., Neves, C.A., Nery, L.E.M., and Trindade, G.S. (2004). Ultraviolet radiation induces dose-dependent pigment dispersion in crustacean chromatophores. Pigment Cell Res. *17*, 545–548.

Hayashi, H., and Fujii, R. (2001). Possible involvement of nitric oxide in signaling pigment dispersion in teleostean melanophores. Zool. Sci. *118*, 1207–1215.

Hermann, A., and Erxleben, C. (2001). Nitric oxide activates voltage-dependent potassium currents of crustacean skeletal muscle. Nitric Oxide *5*, 361–369.

Hill, H.Z., and Hill, G.J. (2000). UVA, pheomelanin and the carcinogenesis of melanoma. Pigment Cell Res. *13*, 140–144.

Hogben, L., and Slome, D. (1931). The pigmentary effector system. IV The dual character of endocrine co-ordination in amphibian color change. Proc. R. Soc. Lond. Ser. B *108*, 10–53.

Horikoshi, T., Nakahara, M., Kaminaga, H., Sasaki, M., Uchiwa, H., and Myiachi, Y. (2000). Involvement of nitric oxide in UVB-induced pigmentation in guinea pig skin. Pigment Cell Res. *13*, 358–363.

Ivanova, K., Le Poole, I.C., Gerzer, R., Westerhof, W., and Das, P.K. (1997). Effect of nitric oxide on the adhesion of human melanocytes to extracellular matrix components. J. Pathol. *183*, 469–476.

Jackson, M., Frame, F., Weller, R., and McKenzie, R.C. (1998). Expression of nitric oxide synthase III (eNOS) mRNA by human skin cells: melanocytes but not keratinocytes express eNOS mRNA. Arch. Dermatol. Res. *290*, 350–352.

Johansson, K.U.I., and Mellon, D. (1998). Nitric oxide as a putative messenger molecule in the crayfish olfactory midbrain. Brain Res. *807*, 237–242.

Kim, H.W., Batista, L.A., Hoppes, J.L., Lee, K.J., and Mykles, D.L. (2004). A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis*. J. Exp. Biol. *207*, 2845–2857.

Konturek, S.K., and Konturek, P.C. (1995). Role of nitric oxid in the digestive system. Digestion *56*, 1–13.

Lachaise, A., Le Roux, A., Hubert, M., and Lafont, R. (1993). The molting gland of crustaceans: localization, activity, and endocrine control (a review). J. Crust. Biol. *13*, 198–234.

Lassalle, M.W., Igarashi, S., Sasaki, M., Wakamatsu, K., Ito, S., and Horikoshi, T. (2003). Effect of melanogenesis-inducing nitric oxide and histamine on the production of eumelanin and pheomelanin in cultured human melanocytes. Pigment Cell Res. *16*, 81–84.

Lee, S.G., and Mybles, D.L. (2006). Proteomics and signaling transduction in the crustacean molting gland. Integr. Comp. Biol. *46*, 965–977.

Lesser, M.P. (2001). Exposure to ultraviolet radiation (290–400 nm) causes oxidative stress, DNA damage, and expression of p53/p73 in laboratory experiments on embryos of the spotted salamander. *Ambystoma maculatum*. Physiol. Biochem. Zool. *74*, 733.

Lipton, S.A., Choi, Y.B., Pan, Z.H., Lei, S.Z., Chen, H.S., Sucher, N.J., Loscalzo, J., Singel, D.J., and Stamler, J.S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature *364*, 626–632.

Lowe, C., and Goodman-Lowe, G. (1966). Suntanning in hammerhead sharks. Nature. *383*, 677.

Lyons, C.R., Orloff, G.J., and Cunningham, J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J. Biol. Chem. *267*, 6370–6374.

Mahadevan, A., Lappé, J., Rhyne, R.T., Cruz-Bermúdez, N.D., Marder, E., and Goy, M.F. (2004). Nitric oxide inhibits the rate and strength of cardiac contractions in the lobster *Homarus americanus* by acting on the cardiac ganglion. J. Neurosci. *24*, 2813–2824.

Marchese, C., Maresca, V., Cardinali, G., Belleudi, F., Ceccarelli, S., Bellocci, M., Frati, L., Torrisi, M.R., and Picardo, M. (2003). UVB-induced activation and internalization of keratinocyte growth factor receptor. Oncogene *22*, 2422–2431.

Marletta, M.A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. Cell *78*, 927–930.

McDonald, L.J., and Murad, F. (1996). Nitric oxide and cyclic GMP signaling. Proc. Soc. Exp. Biol. Med. *211*, 1–6.

Miller, C.C., Hale, P., and Pentland, A.P. (1994). Ultraviolet B injury increases prostaglandin synthesis through a tyrosine kinase-dependent pathway. Evidence for UVB-induced epidermal growth factor receptor activation. J. Biol. Chem. *269*, 3529–3533.

Miranda, K.M., Nims, R.W., Thomas, D.D., Espey, M.G., Citrin, D., Bartberger, M.D., Paolocci, N., Fukuto, J.M., Feelisch, M., and Wink, D.A. (2003). Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS. J. Inorg. Biochem. *93*, 52–60.

Murad, F. (1994). Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cyclic GMP signal transduction system. Adv. Pharmacol. *26*, 19–33.

Murphy, G., Young, A.R., Kulms, H.C., and Schwarz, T. (2001). The molecular determinants of sunburn cell formation. Exp. Dermatol. *10*, 155–160.

Nishida, K., Harrison, D.G., Navas, J.P., Fisher, A.A., Dockery, S.P., Uematsu, M., Nerem, R.M., Alexander, R.W., and Murphy, T.J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial nitric oxide synthase. J. Clin. Invest. *90*, 2092–2096.

Oberg, H., Karlsson, A., Sundqvist, T., and Svensson, S. (1999). Endogenous production of nitric oxide can alter the directed transport of pigment organelles in *Xenopus laevis* melanophores. Pigment Cell Res. *12*, Suppl 7, 68.

Oshima, N. (2001). Direct reception of light by chromatophores of lower vertebrates. Pigment Cell Res. *14*, 312–319.

Paunel, A.N., Dejam, A., Thelen, S. et al. (2005). Enzyme-independent nitric oxide formation during UVA challenge of human skin: characterization, molecular sources, and mechanisms. Free Radic. Biol. Med. *38*, 606–615.

Rocha, I.M., and Guillo, L.A. (2001). Lipopolysaccharide and cytokines induce nitric oxide synthase and produce nitric oxide in cultured normal human melanocytes. Arch. Dermatol. Res. *293*, 245–248.

Romero-Graillet, C., Aberdam, E., Biagoli, N., Massabai, W., Ortonne, J.P., and Ballotti, R. (1996). Ultraviolet B radiation acts through nitric oxide and cGMP signal pathways to stimulate melanogenesis in human melanocytes. J. Biol. Chem. *271*, 28052–28056.

Romero-Graillet, C., Aberdam, E., Clement, M., Ortonne, J.P., and Ballotti, R. (1997). Nitric oxide produced by ultraviolet-irradiated keratinocytes stimulates melanogenesis. J. Clin. Invest. *99*, 635–642.

Sakai, M., Shimizu, Y., Nagatsu, I., and Ueda, H. (1996). Immunohistochemical localization of NO synthases in normal human skin and psoriatic skin. Arch. Dermatol. Res. *288*, 625–627.

Sapura, M., Hiragushi, T., Ohpaware, K., and Aonuma, H. (2006). Expression of NO-cGMP signaling in the olfactory center of the ant *Aphaenogaster smythiese japonica*. Comp. Biochem. Physiol. B *145*, 419–419.

Sasaki, M., Horikoshi, T., Uchiwa, H., and Myiashi, Y. (2000). Up-regulation of tyrosinase gene by nitric oxide in human melanocytes. Pigment Cell Res. *13*, 248–252.

Shimizu, Y., Sakai, M., Umemura, Y., and Ueda, H. (1997). Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. J. Dermatol. *24*, 80–87.

Sirsjo, A., Karlsson, M., Gidlof, A., Rollman, O., and Torma, H. (1996). Increased expression of inducible nitric oxide synthase in psoriatic skin and cytokine-stimulated cultured keratinocytes. Br. J. Dermatol. *134*, 643–648.

Skinner, D.M. (1985). Molting and regeneration. In The Biology of Crustacea, D.E. Bliss, and L.H. Mantel, eds. (New York, NY: Academic Press).pp. 43–146.

Wink, D.A., and Mitchell, J.B. (1998). Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radic. Biol. Med. *25*, 434–456.

Yoshida, M., Saegusa, Y., Fukuda, A., Akama, Y., and Owada, S. (2005). Measurement of radical-scavenging ability in hepatic metallothionein of rat using in vivo electron spin resonance spectroscopy. Toxicology. *213*, 74–80.