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Participation of the NO/cGMP/K⁺ATP pathway in the antinociception induced by Walker tumor bearing in rats

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Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, CE, Brasil

Abstract

Implantation of Walker 256 tumor decreases acute systemic inflammation in rats. Inflammatory hyperalgesia is one of the most important events of acute inflammation. The L-arginine/NO/cGMP/K⁺ATP pathway has been proposed as the mechanism of peripheral antinociception mediated by several drugs and physical exercise. The objective of this study was to investigate a possible involvement of the NO/cGMP/K⁺ATP pathway in antinociception induced in Walker 256 tumor-bearing male Wistar rats (180-220 g). The groups consisted of 5-6 animals. Mechanical inflammatory hypernociception was evaluated using an electronic version of the von Frey test. Walker tumor (4th and 7th day post-implantation) reduced prostaglandin E₂- (PGE₂, 400 ng/paw; 50 µL; intraplantar injection) and carrageenan-induced hypernociception (500 µg/paw; 100 µL; intraplantar injection). Walker tumor-induced analgesia was reversed (99.3% for carrageenan and 77.2% for PGE₂) by a selective inhibitor of nitric oxide synthase (L-NAME; 90 mg/kg, *ip*) and L-arginine (200 mg/kg, *ip*), which prevented (80% for carrageenan and 65% for PGE₂) the effect of L-NAME. Treatment with the soluble guanylyl cyclase inhibitor ODQ (100% for carrageenan and 95% for PGE₂; 8 µg/paw) and the ATP-sensitive K⁺ channel (KATP) blocker glibenclamide (87.5% for carrageenan and 100% for PGE₂; 160 µg/paw) reversed the antinociceptive effect of tumor bearing in a statistically significant manner ($P < 0.05$). The present study confirmed an intrinsic peripheral antinociceptive effect of Walker tumor bearing in rats. This antinociceptive effect seemed to be mediated by activation of the NO/cGMP pathway followed by the opening of KATP channels.

Key words: Walker 246 tumor-bearing rats; Hypernociception; Nitric oxide; Carrageenan

Introduction

Cancer can induce immunosuppression by an immunosuppressive network extending from the primary tumor site to secondary lymphoid organs and peripheral vessels (1). The Walker 256 tumor is widely utilized in experimental cancer research due to its easy transplantation, lack of regression or strain specificity, and rarity of spontaneous metastases (2). Recently, our group demonstrated that Walker 256 tumor implantation limits mast cell function and vascular events in acute inflammation in rats, without changes in neutrophil migration (3).

Inflammatory hyperalgesia results from the sensitization of primary afferent neurons, which is better described as hypernociception (decrease in nociceptive threshold) in animal models (4). This effect is induced by inflammatory mediators, such as prostaglandins (PGs), which directly

sensitize peripheral nociceptive neurons (5-7), and a coordinated cascade of cytokines that precedes the release of other directly acting mediators (8,9). In fact, following the administration of carrageenan in the rat paw, an initial formation of bradykinin occurs, which induces a subsequent proinflammatory cytokine cascade, triggering the release of PGs and sympathetic amines (10).

In inflammatory hypernociception, nitric oxide (NO) activates the guanylate cyclase enzyme, which is directly responsible for an increase in intracellular levels of cyclic guanosine monophosphate (cGMP) (11). Then, cGMP induces the opposite effect of cyclic adenosine monophosphate (cAMP) (12) and promotes antihypernociception. Sachs et al. (13) demonstrated that activation of protein kinase G by cGMP is necessary for the opening of ATP-

Correspondence: M.H.L.P. Souza, Centro de Biomedicina, Faculdade de Medicina, Universidade Federal do Ceará, Rua Cel. Nunes de Melo, 1315, 60430-270 Fortaleza, CE, Brasil. Fax: +55-85-3366-8333. E-mail: souzamar@ufc.br

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sensitive K⁺ channels (KATP) in analgesia. However, the role of the NO/cGMP/K⁺ATP channel signaling pathway in the tumor bearing-induced decrease in systemic acute inflammation in rats has not been elucidated.

The objective of the present study was to investigate a possible involvement of the NO/cGMP/K⁺ATP pathway in the antinociception induced by Walker 256 tumor implantation in rats.

Material and Methods

Animals

Wistar rats weighing 180-200 g were housed in temperature-controlled rooms and received water and food *ad libitum*. All experiments were conducted in accordance with National Institutes of Health standards and approved by the Committee of Ethics in Animal Research and Care of the Federal University of Ceará (protocol No. 10/11).

Drugs

Carrageenan, PGE₂, L-NAME, aminoguanidine, L-arginine, ¹H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and glibenclamide were purchased from Sigma Chemicals (USA). Vehicle solutions consisted of PBS or saline. Glibenclamide was dissolved in vehicle containing saline plus 2% Tween (Sigma). ODQ was dissolved in saline plus 2% dimethyl sulfoxide (Sigma). PGE₂, carrageenan, L-NAME, aminoguanidine, and L-arginine were dissolved in saline.

Tumor implantation

Tumor nodules from Walker 256 tumor-bearing rats were excised, gently homogenized with a hand-operated tissue grinder, suspended in sterile lactated Ringer's solution with gentamicin (20%, v/v) and adjusted to 10⁶ viable tumor cells/mL. The tumor was implanted by weekly intramuscular injections of 10⁶ tumor cells into the left armpit (14). Animals were housed in cages with food/mineral water *ad libitum* and the effects of Walker 256 tumor bearing was investigated after the 4th or 7th day of tumor implantation.

In preliminary experiments, tumor growth was monitored starting on the 4th day and continuing to the 7th day, reaching maximum growth on the 15th day after inoculation. We also observed that 15 days after tumor inoculation, animal mortality reached 100% (3). Thus, in this study, we evaluated the effects of tumor bearing on the 4th and 7th day after tumor inoculation.

Mechanical hypernociception

The term hypernociception (increased nociception) is used to describe the behavioral response induced by mechanical pressure on rats. Hyperalgesia was induced by a subcutaneous injection of carrageenan (500 µg/paw) or PGE₂ (400 ng/paw) into the plantar surface of the rat's hindpaw and measured by the paw pressure test as de-

scribed by Cunha et al. (15). In the test, the investigator was trained to apply the filaments or the polypropylene tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The test consisted of poking a hindpaw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. Each von Frey filament was applied for approximately 3-4 s to induce the end-point reflex. The weakest filament able to elicit a response was taken to be the mechanical threshold (g).

A digital analgesiometer (Insight, Brazil) with a cone-shaped paw-presser with a rounded tip was used to apply a linearly increasing force to the rat's right hindpaw. The nociceptive threshold was measured in the right paw and determined by the average of three consecutive trials recorded before (zero time) and 3 and 4 h after carrageenan (100 µL; 500 µg/paw) or PGE₂ (50 µL; 400 ng/paw) injection (peak effect). Hyperalgesia was calculated as the difference between these two averages (Δ of nociceptive threshold) and is reported in grams. To reduce stress, the rats were habituated to the apparatus one day prior to the experiments (15).

Role of Walker tumor implantation on carrageenan- or PGE₂-induced hypernociception

Four or 7 days after Walker tumor implantation in the left armpit, carrageenan- or PGE₂-induced mechanical hypernociception was evaluated in the contralateral paw. The control group received only saline in the left armpit. The nociceptive threshold was measured in the right paw and determined as the average of three consecutive trials recorded before (zero time) and 3 and 4 h after carrageenan (100 µL; 500 µg/paw) or PGE₂ (50 µL; 400 ng/paw) injection (peak effect).

Role of NO in the antinociceptive effect induced by Walker tumor implantation

Four days after Walker tumor implantation in the left armpit, rats were treated with L-NAME (a nonselective nitric oxide synthase (NOS) inhibitor, 90 mg/kg; 0.5 mL; *ip*) or aminoguanidine (an inducible NOS (iNOS) selective inhibitor, 10 mg/kg; 0.2 mL; subcutaneous injection). After 1 h, carrageenan or PGE₂ was administered to the right paw. The other experimental group was treated in the same way, but L-arginine (an NOS substrate, 200 mg/kg; 0.5 mL; intraperitoneal injection) was given 10 min before L-NAME administration. In the control group of rats without tumors, L-NAME alone was also injected before PGE₂ or carrageenan administration. In all experimental groups, the nociceptive threshold was measured in the right paw and determined as described above.

Role of cGMP in antinociceptive effect induced by Walker tumor implantation

Four days after Walker tumor implantation in the left armpit, the soluble guanylyl cyclase inhibitor ODQ (8 µg/

paw; 50 μ L; intraplantar injection) or 2% Tween (diluent) was injected. After 30 min, carrageenan or PGE₂ was administered to the right paws of rats. In other groups of rats without tumors, ODQ alone was also injected before carrageenan or PGE₂ administration. The nociceptive threshold was then measured in the right paw and determined as described above.

Role of KATP in the antinociceptive effect induced by Walker tumor implantation

Four days after Walker tumor implantation in the left armpit, glibenclamide (a KATP blocker, 160 μ g/paw; 50 μ L; intraplantar injection) or dimethyl sulfoxide (diluent) was injected 30 min before carrageenan and PGE₂ administration in the right paws of rats. In the control group of rats without tumors, glibenclamide alone was also injected before carrageenan or PGE₂ administration. The nociceptive threshold was then measured in the right paw and determined as described above.

Statistical analysis

Data are reported as means \pm SEM for groups of 5 or 6 animals each. Differences between experimental groups were compared by analysis of variance (ANOVA) followed by the Bonferroni *t*-test. The level of significance was set at $P < 0.05$.

Results

Role of Walker tumor implantation in carrageenan- or PGE₂-induced hypernociception

Figure 1 shows that on the 4th or 7th day after tumor implantation there were significant decreases in both carrageenan- (Panel A) and PGE₂-induced (Panel B)

hypernociception compared to the control group of rats without tumors.

Involvement of NO in the antinociceptive effect induced by Walker tumor implantation

Figure 2 shows that pretreatment with L-NAME alone completely reversed the antinociceptive activity of Walker tumor at both 3 and 4 h after treatment with carrageenan (Panel A, 3 h = 99.3%, 4 h = 100%) or PGE₂ (Panel B, 3 h = 77.2%, 4 h = 95.7%). In the control animals without tumors, L-NAME did not increase the inflammatory hypernociception induced by carrageenan (Panel A) or PGE₂ (Panel B). L-arginine was able to reverse the effect of L-NAME on inflammatory hypernociception induced by carrageenan (Panel A) or PGE₂ (Panel B). On the other hand, aminoguanidine treatment was not able to change the antinociceptive effect of tumor bearing in PGE₂-induced hypernociception (Panel B).

Involvement of cGMP in the antinociceptive effect induced by Walker tumor implantation

In the carrageenan- (Figure 3, Panel A) or PGE₂-induced (Panel B) mechanical hypernociception, ODQ treatment completely reversed Walker tumor-induced antinociceptive activity at both 3 and 4 h ($P < 0.05$). In the control animals without tumors, ODQ did not increase the inflammatory hypernociception induced by either carrageenan (Panel A) or PGE₂ (Panel B).

Involvement of KATP in the antinociceptive effect induced by Walker tumor implantation

Figure 4 shows that pretreatment with the specific KATP blocker glibenclamide significantly inhibited Walker tumor-induced antinociceptive activity at both 3 and 4 h

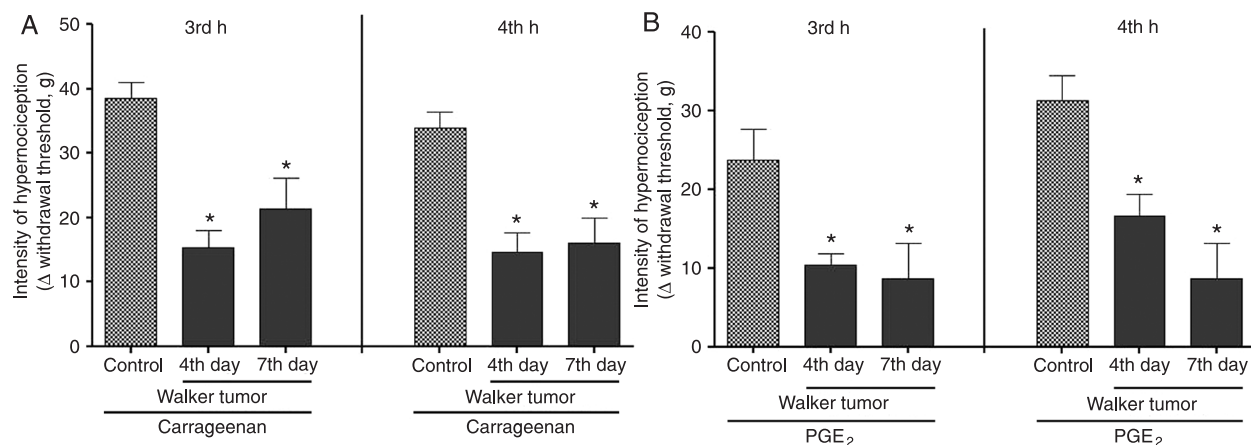


Figure 1. Effect of Walker tumor implantation on carrageenan- or prostaglandin E₂ (PGE₂)-induced hypernociception. Carrageenan (500 μ g/paw) and PGE₂ (400 ng/paw) were injected and paw hypernociception was measured after 3 and 4 h for carrageenan (Panel A) and PGE₂ intraplantar injection (Panel B). * $P < 0.05$ compared to saline group (ANOVA/Bonferroni test).

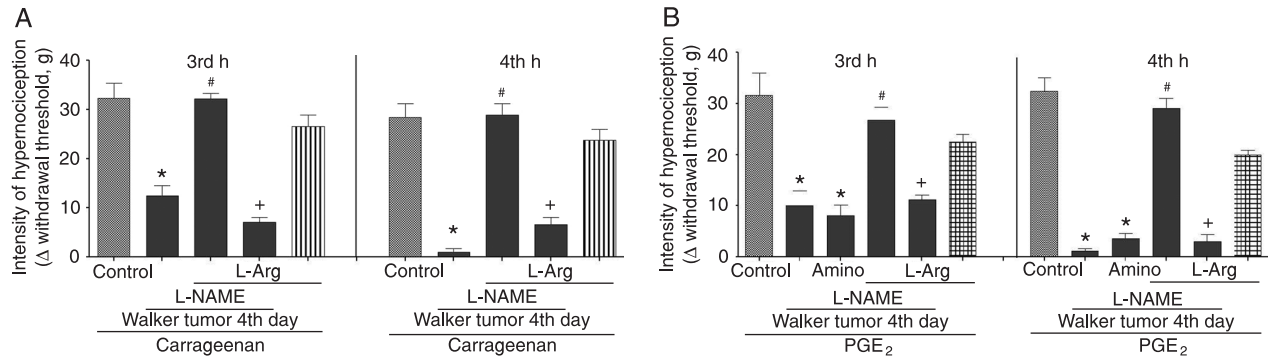


Figure 2. Involvement of nitric oxide in the antinociceptive effect induced by Walker tumor implantation. N^{ω} -nitro-L-arginine methyl ester (L-NAME; 90 mg/kg; 0.5 mL) or aminoguanidine (10 mg/kg; 0.2 mL) was administered 1 h before prostaglandin E_2 (PGE_2) or carrageenan administration in the right paws of rats with (4th day after tumor implantation) or without tumor. L-arginine (L-Arg; 200 mg/kg; 0.5 mL) was administered 10 min before L-NAME injection. The analgesic activity of Walker tumors in the right hind paw was measured 3 or 4 h after carrageenan- (Panel A) or PGE_2 -induced hypernociception (Panel B). * $P < 0.05$ compared to saline group; # $P < 0.05$ compared to Walker tumor group; * $P < 0.05$ compared to Walker tumor + L-NAME group (ANOVA/Bonferroni test).

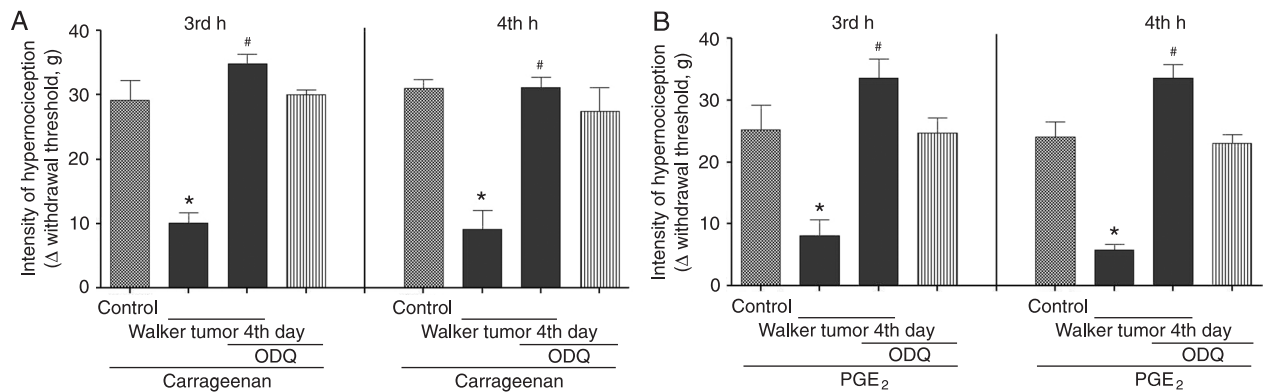


Figure 3. Involvement of cGMP in the antinociceptive effect induced by Walker tumor implantation. 1H -[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (8 μ g/paw; 50 μ L) was administered 30 min before carrageenan (500 μ g/paw; 100 μ L) or prostaglandin E_2 (PGE_2 ; 400 ng/paw; 50 μ L) administration in the right paws of rats with (4th day after tumor implantation) or without tumor. The analgesic activity of Walker tumors in the right hind-paw was measured 3 or 4 h after carrageenan (Panel A) or PGE_2 -induced hypernociception (Panel B). # $P < 0.05$ compared to Walker tumor group. * $P < 0.05$ compared to saline group (ANOVA/Bonferroni test).

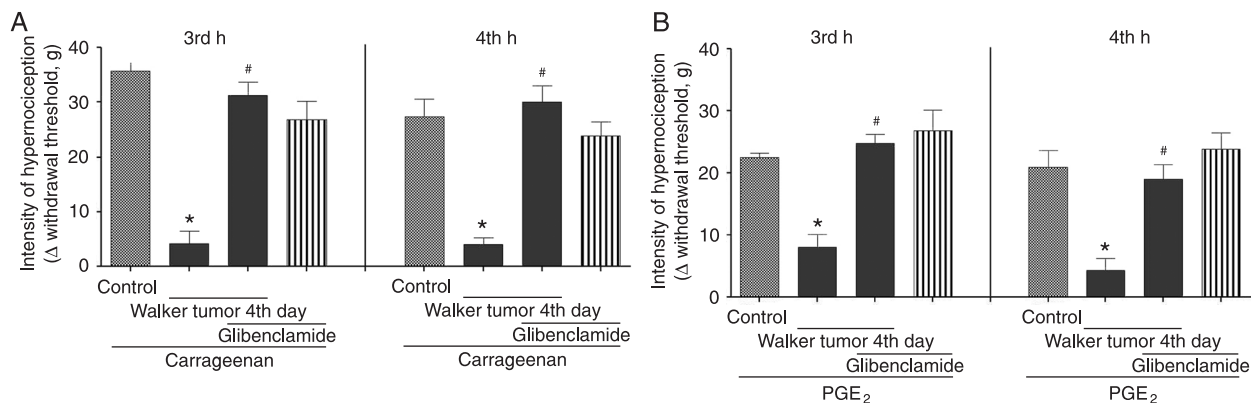


Figure 4. Involvement of ATP-sensitive K^+ (KATP) channels in the antinociceptive effect induced by Walker tumor implantation. Glibenclamide (160 μ g/paw) was administered 30 min before carrageenan (500 μ g/paw; 100 μ L) or prostaglandin E_2 (PGE_2 ; 400 ng/paw; 50 μ L) administration to rats with (4th day after tumor implantation) or without tumor. The analgesic activity of Walker tumors in the right hind-paw was measured 3 or 4 h after carrageenan- (Panel A) or PGE_2 -induced (Panel B) hypernociception. # $P < 0.05$ compared to Walker tumor group. * $P < 0.05$ compared to saline group (ANOVA/Bonferroni test).

after treatment with carrageenan (Panel A, 3 h = 87.5%, 4 h = 100%) or PGE₂ (Panel B, 3 h = 100%, 4 h = 85.6%). In the control animals without tumors, glibenclamide did not increase the inflammatory hypernociception induced by either carrageenan (Panel A) or PGE₂ (Panel B).

Discussion

The influence of the tumor microenvironment on the acute systemic inflammatory response has not been clearly elucidated. Recently, our group demonstrated that Walker tumor bearing can limit mast cell function and vascular events in acute systemic inflammation models in rats, without changes in neutrophil migration (3). In the present study, our results demonstrate that Walker tumor bearing produced a peripheral antinociceptive effect in PGE₂- or carrageenan-induced mechanical hypernociception, and this effect was mediated at least in part by activation of the NO/cGMP pathway, followed by the opening of KATP channels.

The induction of inflammatory hypernociception is primarily driven by the sensitization of primary nociceptive neurons, an effect that is caused by nociceptor-sensitizing mediators. Our results demonstrated that Walker tumor implantation in the contralateral paw decreased both carrageenan- and PGE₂-induced hypernociception. Carrageenan-induced hypernociception depends on the concomitant release and action of cytokines, which induces the subsequent release of IL-1 β /prostanoids (16). On the other hand, PGE₂, stimulated by cytokines, acts on nociceptive neurons by a direct mechanism (16-18). One possibility was that tumor bearing decreased the inflammatory response by decreasing cytokine release and, subsequently, inflammatory hypernociception. This hypothesis is consistent with our recent results, which demonstrated that Walker tumor bearing reduced carrageenan-induced paw edema (3). Published reports have demonstrated that the late edema phase induced by carrageenan depends on cytokine production by resident cells and neutrophil infiltration (10,18). However, in our opinion, it is not the only mechanism involved in the tumor bearing-induced decrease in inflammatory hypernociception, as PGE₂-induced hypernociception

was also decreased after Walker tumor implantation. We can infer that tumor bearing decreases both inflammation and hypersensitivity of nociceptive neurons.

We also provide evidence in the present study for the molecular mechanism by which tumor bearing promotes analgesia, i.e., stimulation of the NO/cGMP/K⁺ATP pathway. Our results clearly demonstrate that pretreatments with a nonspecific iNOS (L-NAME), guanylyl cyclase inhibitor (ODQ) and KATP blocker (glibenclamide) all reversed the antinociceptive effect induced by tumor bearing, suggesting that the NO/cGMP/K⁺ATP pathway plays a role in this response. The peripheral analgesic activity of the NO/cGMP pathway seems to result from the modulation of KATP currents. For instance, the peripheral antinociceptive effects of opioids, NO donors and cGMP are inhibited by KATP blockers (19-22). Our findings agree with literature reports that have demonstrated reversal of antinociceptive effects after inhibition of the NO/cGMP/K⁺ATP pathway (23-25).

Although we did not address the mechanism by which tumor bearing stimulates the peripheral NO analgesic pathway, there is evidence that systemic immunosuppression and angiogenic stimuli associated with malignant tumor growth could be mediated in part by mechanisms dependent on NO overproduction (26). This strengthens the hypothesis that the NO/cGMP/K⁺ATP pathway may be involved in tumor bearing-induced analgesia.

The present study showed an intrinsic peripheral antinociceptive action of Walker tumor bearing in rats. This antinociceptive effect seemed to be mediated by activation of the NO/cGMP pathway followed by the opening of KATP channels. This information may contribute to a better understanding of the effect of the tumor microenvironment on the inflammatory process, which could elucidate how malignant tumor growth can regulate nociceptive neurons, as well as tumor progression and invasion.

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