Indian Journal of Experimental Biology
Vol. 61, May 2023, pp. 349-356
DOI: 10.56042/ijeb.v61i05.854

Role of NO/cGMP/kATP pathway on diosgenin induced antinociceptive activity by formalin and hotplate induced model in rats

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Received 29 October 2022; *revised 01 March 2023*

Diosgenin is a natural steroid sourced from plants and exhibits good analgesic and anti-inflammatory activity. However, mechanism of action associated with the analgesic activity of diosgenin is not yet delineated. Hence, in the present study, we explored the mechanism of analgesic activity of diosgenin on the nitric oxide/cyclic guanosine monophosphate/adenosine monophosphate sensitive potassium channels (NO/cGMP/kATP). The role of (NO/cGMP/kATP) in the Wistar rat was studied using formalin-induced models and hot plate models with various antagonists, such as N-nitro-L-arginine methyl ester hydrochloride (L NAME), glibenclamide, 1H-(1,2,4) oxadiazole (4,3-A) quinoxaline-1-one (ODQ) and 7-nitroindazole. Two doses of diosgenin were used in the study (25 and 50 mg/kg). Diosgenin reached its maximal effect (P <0.001) at (50 mg/kg po) in formalin as well as a hot plate induced nociception. This study has demonstrated that prior administration of the selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (0.1-1 mg/kg i.p.), glibenclamide, an ATP K^+ channel inhibitor, L-NAME (10 mg/kg i.p.) and ODQ (2 mg/kg i.p.) significantly prevents the antinociceptive effect of diosgenin in formalin and hot plate induced nociception (50 mg/kg i.p administered 10 min after). It suggests that NO/cGMP/kATP has a significant role in the antinociceptive activity of diosgenin.

Keywords: Analgesic, Fenugreek, Inflammation, Paw oedema, Potassium channels

Nitric oxide (NO) is involved in multiple physiological functions and also in the pathophysiology of diseases. It has a well-documented role in peripheral antinociceptive activity[1]. The soluble guanylyl cycles enzyme is stimulated by NO released at the site of injury, which raises the cellular concentration of cyclic guanosine monophosphate (cGMP)[2]. The subsequent activation of L-arginine and NO/cGMP opens the ATP-sensitive potassium channels (kATP), which play a role in antinociceptive activity[3]. The antinociceptive activity of NO, cGMP, and KATP in the peripheral nervous system has been well established in numerous studies[4], as the role of potassium channels and potassium currents in the regulation of neuronal excitability. Potassium ions decrease the membrane potential charge and decrease the release of neurotransmitters[5]. This pathway was also described for peripheral antinociceptive mechanism of the opioids ketorolac and ibuprofen[6]. The studies prove that potassium channel blockers decrease the antinociceptive response of morphine in a dose-dependent manner when administered intravenously[7].

On the other hand, studies also prove that potassium channel openers intensify the anti-nociceptive activity of morphine[8]. NO/cGMP and KATP have a role in regulating the antinociceptive effect of central and peripheral analgesic drugs[9].

Diosgenin, a naturally occurring steroidal saponin, is a major active ingredient in varied edible roots and pulses. It is found in abundance in the seeds of fenugreek (*Trigonella foenum graecum* Linn.) and in the root tubers of wild yams (*Dioscorea villosa* Linn.)[10]. China and Mexico are the two countries with the richest yam resources in the world, and diosgenin yields account for 85% of the world's production[10] disogenin has a wide variety of biological activities; it decreases the absorption of cholesterol and prevents its accumulation in the liver cells[11], and it was identified as a potential compound against COVID 19 virus[12].

It shows significant anti-inflammatory and analgesic activity in carrageenan induced paw edoema

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model[6,13] but the mechanism underlying this action is not clearly known. Therefore, in the present study, we investigaed different mechanisms involved in the antinociceptive effect of diosgenin using different antagonists, particularly the L-arginine/NO/cGMP/ KATP channel pathway.

Materials and Methods

Animals

The studies were done on adult male Wistar rats (200-220 g), which were acquired from PSG- Institute of Medical Sciences and Research (IMSR), Coimbatore breeding facilities. The experimental animals had free access to food and water before the study. The animals were housed at 22±2ºC under a 12 h light/dark cycle. They were acclimatized to the laboratory for at least 12 h before testing. The research was performed after the review and approval of the protocol by the institution's Animal Ethics Committee of PSG-IMSR (277/2015/IAEC) and was carried out in accordance with current laboratory animal care guidelines and ethics for the investigation of experimental pain in conscious animals[14]. The experiments were performed between 9 a.m. and 5 p.m.

Preparation of drugs and chemicals

All chemicals used in the experiment were obtained from Sigma Aldrich. L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride) was dissolved in a 0.9% saline solution. Glibenclamide (5-chloro-N-[4-(cyclohexyl ureido sulfonyl) phenethyl]-2-methoxy-benzamide) was dissolved in 5% DMSO. ODQ (1H-(1,2,4) oxadiazole (4,3-A) quinoxaline-1-one) was dissolved in 50% DMSO. 7-nitroindazole (7-nitro-1H-indazole) was dissolved in 10% Tween 20.

Dose optimization of Diosgenin

Four groups of animals were used for dose optimization and each group contained 6 animals, three doses were used for optimizing the dose of diosgenin (25, 50 and 100 mg/kg) and one group served as a control. The dose was optimized by formalin induced paw licking and paw elevation behaviour in both phases[15].

Formalin test

Twelve groups of animals were used in the study having 6 animals in each. After the acclimatization period, the rats were given diosgenin (25 and 50 mg/kg) and vehicle 1 h prior to the formalin test, which consisted of administering 50 µL of formalin[16] (2.5% in normal saline) in the sub-plantar region of the hind paw of rats. The animals were placed in an individual observation chamber after the formalin administration and antinociceptive activity was measured by calculating the paw flinching/licking and paw elevation[17] times during the first 10 min (acute/neurogenic phase) and at 20-40 min (delayed/ inflammatory phase)[18] group are summarised in Table 1.

Hot plate test

The hot plate test was carried out to measure the latency time of the rat submitted to the hot plate, as previously described earlier[19]. In the thermal nociceptive test, the reaction time that elapsed between contact with the hot plate at 55±1.0°C and jumping or paw licking in response to pain was measured in seconds with a cut-off time of 30 s. The measurements were taken at the beginning and 50 min after the drug was administered[9].

To evaluate the possible mechanisms involved in the antinociceptive action of diosgenin, rats were treated with different doses of antagonistic drugs that were administered IP 10 min before administration of an optimal dose of diosgenin (50 mg/kg p.o.) that was given 60 min prior to formalin injection. These substances and their dosages were chosen using information from scientific literature and previous experiments[6]. To evaluate the involvement of nNOS/NO, we used the non-selective neuronal nitric oxide synthase inhibitor, L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride) (1-10 mg/kg, i.p.) and 7-nitroindazole (7-nitro-1H-indazole) (0.1–1 mg/kg, i.p.), a selective neuronal nitric oxide synthase inhibitor[17].

Open field

This test was carried out to rule out the possibility that the antinociceptive effect of diosgenin was due to

Table 1 — Treatment protocols followed in the study

Group	Treatment	Respective dose
I	Control	NS
II	Diosgenin	(50 mg/kg p.o)
III	Diosgenin + 7-Nitroindazole	(50 mg/kg p.o)+(0.1 mg/kg i.p.)
IV	Diosgenin + 7-Nitroindazole	(50 mg/kg p.o)+(1 mg/kg i.p.)
V	7-Nitroindazole	(1 mg/kg i.p.)
VI	Diosgenin + Glibenclamide	(50 mg/kg p.o)+(1 mg/kg i.p.)
VII	Diosgenin + Glibenclamide	(50 mg/kg p.o)+(10 mg/kg i.p.)
VIII	Glibenclamide	(10 mg/kg i.p.)
IX	Diosgenin + L-NAME	(50 mg/kg p.o)+(1 mg/kg i.p.)
X	Diosgenin + L-NAME	(50 mg/kg p.o)+(10 mg/kg i.p.)
XI	L-NAME	(10 mg/kg i.p.)
XII	Diosgenin + ODQ	(50 mg/kg p.o)+(0.2 mg/kg i.p.)
XIII	Diosgenin + ODQ	(50 mg/kg p.o)+(2 mg/kg i.p.)
XIV	ODQ	(2 mg/kg i.p.)

non-specific modifications of the animals' locomotive activity by assessing their ambulatory behaviour. It was carried out according to the previous descriptions[17]. The apparatus consisted of a transparent sided box measuring 60×60×45 cm. The floor of the field is divided into 9 identical squares. Rats were treated for 1 h beforehand. The time spent in the central compartment, grooming time, immobility time, number of ambulations with all four paws crossing, number of rearing, and number of faeces were observed within a period of 5 min.

Statistical analysis

The values were expressed as mean± S.E.M.). The statistical analysis was performed using one-way ANOVA, followed by post hoc Tukey's multiple comparison tests. Values are expressed as the mean ± SD. for six animals. The P value of 0.05 was deemed significant.

Results

Dose optimization of diosgenin

Pretreatment (for 1 h) with diosgenin (25, 50 and 100 mg/kg o.p.) reduced the formalin-induced paw licking and paw elevation behaviour in both the first and second phases of nociception. The maximal effect of diosgenin was attained at dose of 50 mg/kg PO, which was highly significant statistically (P <0.001) compared to the control group in the acute phase as well as in the delayed phase of nociception. There was no significant increase in the antinociceptive with an increase in dose level (Fig. 1B)

Formalin test

Effect of 7-nitroindazole treatment on acute phase

Prior administration of the selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (0.1 mL and 1 mg/kg i.p.) prevented the antinociceptive effect of diosgenin (50 mg/kg i.p. administered (10 min later) measured as the latency of paw licking or elevation in a highly significant manner (P <0.001) in both the doses that it was used (0.1 and 1 mg/kg i.p. 7-nitroindazole (1 mg/kg i.p.) treated group showed a non-significant but slight increase in paw licking/elevation behaviour when compared to the control group (Fig. 2A).

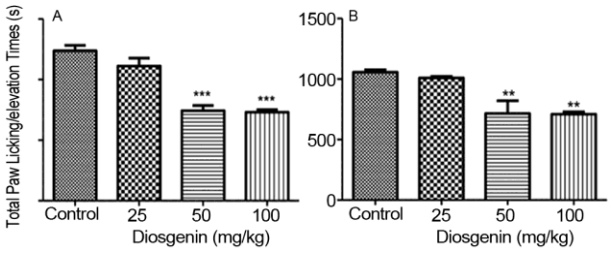

Fig. 1 — Effect of diosgenin (25, 50, 100 mg/kg) in (A) acute phase; and (B) delayed phase (0-10 min) of formalin test. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test. [Values are expressed as the mean ±SD for 6 animals. ** and *** denote statistical significance of disogenin compared to control group at P <0.01 and P <0.001, respectively]

Effect of 7-nitroindazole treatment on delayed phase

The 7-nitroindazole (1 mg/kg i.p.) significantly (P <0.01) reduced the antinociceptive effect of diosgenin (50 mg/kg p.o.) when administered 10 min prior to dosing with diosgenin. No drug significantly changed the paw licking or elevation when compared with the control group that did not receive any drug, indicating that it did not induce nociception or inhibit it. (Fig. 2B).

Effect of glibenclamide on acute phase

Glibenclamide, an ATP-K+ channel inhibitor, at 1 mg/kg IP did not prevent the antinociceptive effect of diosgenin (50 mg/kg p.o.) when given 10 min before glibenclamide. Whereas when administered at a dose of 10 mg/kg i.p., it inhibited the nociceptive action of diosgenin in a highly significant manner (P <0.001) when compared to the group administered diosgenin (50 mg/kg po) alone. In comparison to the control group, the glibenclamide (10 mg/kg i.p.) alone treated group showed a non-significant minute reduction in paw licking or elevation (Fig. 3A).

Effect of glibenclamide on delayed phase

The group that received glibenclamide (10 mg/kg i.p.) 10 min before receiving diosgenin (50 mg/kg p.o.) showed statistically significant (P <0.05) inhibition of the effect of diosgenin when compared to the group that received only diosgenin (50 mg/kg p.o.). In comparison to the control group, the group

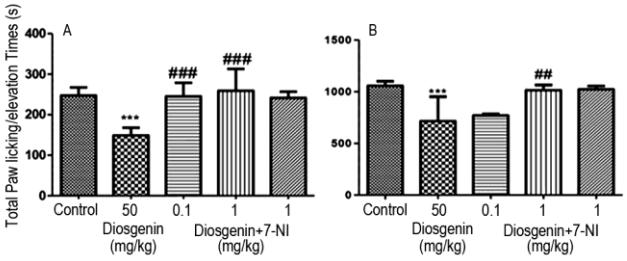

Fig. 2 — Effect of 7-nitroindazole (0.1-1 mg/ kg) on anti-nociceptive activity of diosgenin in (A) acute phase; and (B) delayed phase (0-10 min) of formalin test. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test .values are expressed as the mean ±SD for 6 animals. *** denotes (P <0.001) *vs*. control group and ## and ### denote (P <0.01 and P <0.001) *vs*. diosgenin 50 mg+0.1 mg / kg 7-nitroindazole and 50 mg +1 mg/kg 7-nitroindazole, respectively]

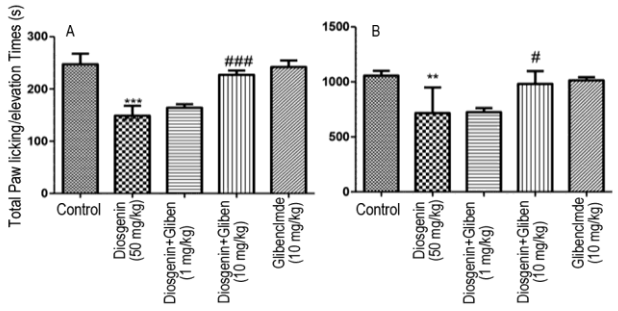

Fig. 3 — Effect of glibenclamide (1-10 mg/kg) on anti-nociceptive activity of diosgenin in (A) acute phase; and (B) delayed phase (0-10 min) of formalin test. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test. [V alues are expressed as the mean ±SD for 6 animals. *** denotes (P <0.001) *vs.* control group. ## and ### denote (P <0.01 and P <0.001) *vs.* diosgenin 50 mg+1 mg/kg glibenclamide and 50 mg +10 mg/kg glibenclamide, respectively]

given glibenclamide (10 mg/kg i.p.) alone showed a non-significant minute reduction in paw licking or elevation (Fig. 3B).

Effect of L NAME treatment on acute phase

Pretreatment with L-NAME (10 mg/kg i.p) a non-selective nitric oxide synthase inhibitor 10 min before diosgenin (50 mg/kg p.o) administration significantly inhibited (P <0.001) antinociceptive effect of diosgenin whereas there was no significant antinociceptive effect of L-NAME at a dose of (1 mg/kg i.p) L-NAME (10 mg/kg i.p) alone showed non-significant inhibition compared to control group (Fig. 4A).

Effect of L NAME treatment on delayed phase

L-NAME (10 mg/kg i.p.) given 10 min before diosgenin (50 mg/kg p.o.) showed a statistically significant (P <0.01) inhibition of the antinociceptive effect when compared to the group that only received diosgenin (50 mg/kg p.o.), whereas L-NAME (1 mg/kg i.p.) did not show a significant level of inhibition. L-NAME (10 mg/kg i.p.) alone showed non-significant inhibition compared to the control group (Fig. 4B).

Effect of ODQ treatment on acute phase

ODQ (2 mg/kg i.p.) administered 10 min before diosgenin (50 mg/kg p.o.) inhibited the antinociceptive action of diosgenin in a highly significant manner (P <0.001), as compared to the nociceptive activity observed in the group treated with only diosgenin (50 mg/kg o.p.). ODQ (0.2 mg/kg I.P.) showed a non-significant reduction of the diosgenin effect under the same conditions. ODQ (10 mg/kg i.p.) alone treated the group, which showed non-significant action compared to the control group (Fig. 5A).

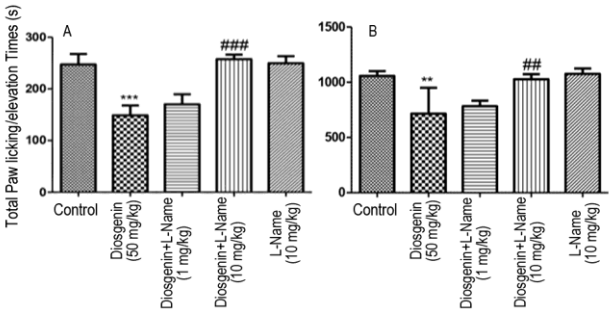

Fig. 4 — Effect of L-NAME (1-10 mg/kg) on anti-nociceptive activity of diosgenin in (A) acute phase (0-10 min); and (B) delayed phase (20-40 min) of formalin test. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test, and are expressed as the mean ±SD for 6 animals. ** and *** denotes (P <0.001) *vs.* control group. ## and ### denote (P <0.01 and P <0.001) *vs.* diosgenin 50 mg+1 mg / kg L-NAME and 50 mg +10 mg/kg L- NAME, respectively

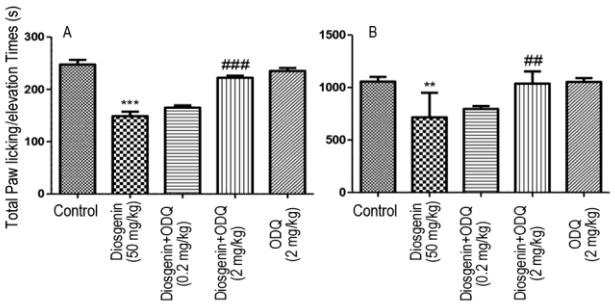

Fig. 5 — Effect of ODQ (0.2-2 mg) on antinociceptive activity of diosgenin in (A) acute phase; and (B) delayed phase (0-10 min) of formalin test. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test, and are expressed as the mean ±SD for 6 animals. ** and *** denotes (P <0.001) *vs.* control group. ## and ### denote (P <0.01 and P <0.001) *vs.* diosgenin 50 mg+0.2 mg/kg ODQ and 50 mg +2 mg/kg L- NAME, respectively]

Effect of ODQ treatment on delayed phase

When compared to the diosgenin (50 mg/kg p.o.) alone treated group, ODQ (2 mg/kg i.p.) showed statistical significance of (P <0.01) inhibition of diosgenin (50 mg/kg o.p.) administered 10 min later. Under the same conditions, ODQ (0.2 mg/kg i.p.) showed non-significant inhibition of diosgenin activity. ODQ (10 mg/kg i.p.) in the treated group alone showed non-significant action compared to the control group (Fig. 5B).

Hot plate test

Diosgenin (25, 50 and 100 mg/kg p.o.) treated groups were effective (exhibited as an increase in the escape latency time) when compared to the control group, reaching maximal antinociceptive effect (at a dose of 50 mg/kg p.o.) with a statistically significant difference (P <0.001). A greater dose of diosgenin

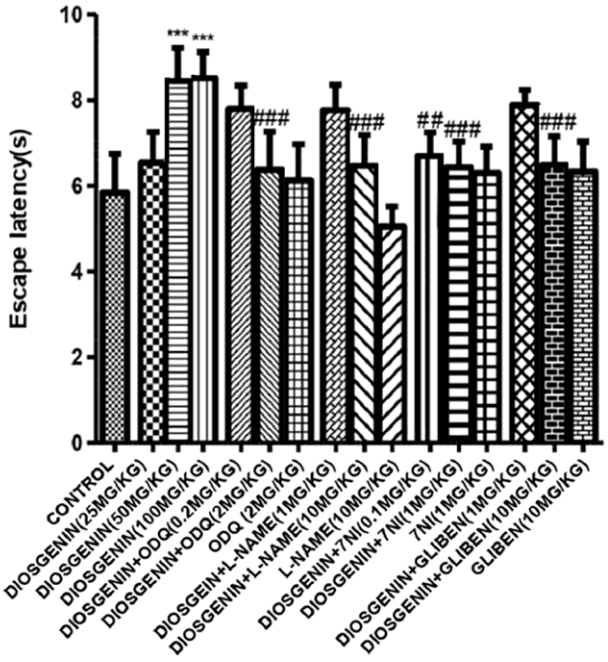

Fig. 6 — Effect of different antagonist of NO/cGMP/kATP on diosgenin to its anti-nociceptive activity using hot plate. [Values are analyzed by one way ANOVA followed by post Tukey's multiple comparison test, and are expressed as the mean ±SD for 6 animals. ** and *** denotes (P <0.01 and P <0.001) *vs*. control group. ## and ### denote (P <0.01 and P <0.001) *vs*. diosgenin 50 mg/kg + different antagonist.

(100 mg/kg p.o.) showed a similar antinociceptive effect as compared to the diosgenin (50 mg/kg p.o.) dose (Fig. 6). When compared to the diosgenin (50 mg/kg p.o.) treated group, groups of different drugs administered 10 min prior to diosgenin (50 mg/kg p.o.) dose dependently prevented the antinociceptive effect of diosgenin (exhibited as a decrease in the escape latency time) with a statistical significance of (P <0.001) for the ODQ (2 mg/kg i.p.) treated group, (P <0.00)

Open field test

Administration of the diosgenin (50 mg/kg p.o.) group showed the same pattern in locomotor activity as the control group. The statistical analysis did not show any significant difference in the observed open field, and hence, the data is not shown.

Discussion

The formalin test involves continuous pain generation from the injured tissue[17] and is a useful method not only for assessing the effects of antinociceptive drugs but also for assisting in the interpretation of the mechanism of action[18]. The neurogenic phase (acute phase) in the formalin test is probably due to the release of substance P by the peripheral stimulation in the paw and centrally mediated pain through C fibre activation. The second phase appears to be dependent on the release of histamine, serotonin, bradykinin, and prostaglandins[19]. Diosgenin inhibited both the first and second phases of formalin-induced pain (P <0.001 and P <0.01, respectively). In the neurogenic phase (Fig. 1A), there was no significant effect of the 25 mg/kg dose on neurogenic-induced pain; only the 50 and 100 mg/kg doses significantly blocked neurogenic pain when compared to the untreated group. The 100 and 50 mg/kg doses showed no significant difference in activity. This result shows the maximum analgesic effect of diosgenin at a dose of 50 mg/kg. In the second phase, inflammatory pain, 50 and 100 mg/kg diosgenin significantly reduced licking time compared to the control group (Fig. 1B). Thus, diosgenin was able to block two phases of the formalin response, but the effect was more noticeable in the initial phase at a dose of 50 mg/kg. The above findings support diosgenin's role in inhibiting prostaglandin synthesis, blocking pro-inflammatory cytokines, and playing a role in the central antinociceptive pathway[20].

The mechanism of the antinociceptive effect of diosgenin is unknown[21]. However, considering that the role of the NO/cGMP/KATP channel pathway plays a vital role in the antinociception of several drugs in the formalin test[22], its possible participation in diosgenin's antinociceptive activity has been investigated in the present study. We discovered that pre-treatment with L-NAME, 7-nitroindazole, ODQ and glibenclamide (for 10 min) prior to diosgenin administration (for 1 h) prior to formalin injection into the paw was effective in modifying the antinociceptive effect of diosgenin (50 mg/kg p.o.) in both phases, indicating the role of NO, cGMP and KATP involvement[23,24]. There was a significant dose-dependent reduction in the anti-nociceptive effect of diosgenin observed in animals after the intraplantar administration of L-NAME (a non-selective inhibitor of the NOS) in the two phases of the formalin test. L-NAME alone, administered at 10 mg/kg, did not produce any effect on formalin-induced nociception as compared with the control group. This proves that L-NAME has no role in hyperalgesic or analgesic effects, and the effect of diosgenin may be partially due to NO modulation (Fig. 4 A and B).

The 7-nitroindazole (a selective neuronal nitric oxide synthase inhibitor) caused maximal reduction

in the diosgenin induced antinociception after intraplantar injection in both phases, with more effect on the acute phases at the lowest dose (0.1 mg/kg), while in the second phase only the higher dose (1 mg/kg) shows significant inhibition. When applied alone, 7-nitroindazole did not produce any significant changes to the formalin-induced nociception as compared to the control group, showing that it had less or no nociceptive or antinociceptive effect and that its effect on diosgenin is partially due to NO modulation (Fig. 2 A and B).

The physiological responses of NO receptors are based on the formation of a soluble form of guanylate cyclase (GC) and the major action of NO is to activate this[25]. Activation of GC increases the conversion of GTP to cGMP and thereby increases the intercellular second messenger level in the tissue and produces antinociceptive action[26]. There were no significant changes observed in ODQ (a GC inhibitor) at a dose of 2 mg/kg i.p. alone to formalin-induced nociception. ODQ reverses both phases of the peripheral antinociceptive effect of diosgenin in a dose-dependent manner. The result obtained from the study shows that NO has a role in the diosgenin induced antinociception because the formalin-induced nociception was not blocked by ODQ[27]. The role of cGMP, a product of GC, in the antinociceptive effect of diosgenin was proven.

The role of KATP channels in peripheral antinociception was well established[23], and the relation between the NO/cGMP pathways for the activation of K channels was also proven[28]. This was established by the study on NO donors and dibutyryladenosine 3,5'-cyclic monophosphate (DbcGMP), KATP channel opener that mediates through a membrane permeable analogue of cGMP[29]. The results reported in our study claim that the manipulation of KATP channels may be an important step in the central mechanism of the antinociceptive effect of diosgenin. Local peripheral administration of glibenclamide (a KATP channel blocker) significantly reduced the antinociceptive action of diosgenin in a dose-dependent manner in both phases, suggesting that diosgenin activates these channels on central and peripheral sites (Fig. 3 A and B).

The NO/cGMP/KATP channel pathway

The connection of the NO/cGMP-KATP channel pathway to the nociceptive process has been proposed[30]. Potassium channels have been shown to play a role in antinociceptive mechanisms[31,32] and to regulate neuronal excitability by allowing K+ ions to pass through the membrane. As a result, membrane potentials are pushed further (hyperpolarization), which decreases the depolarization and action potential transmission capacities of neurons, thereby prompting analgesia[33].

This produced nitric oxide activates guanylate cyclase-C (GC-C)[34], a type 1 transmembrane receptor that initiates the consequent effects of endogenous hormones guanylin and uroguanylin (cyclic guanosine-3′,5′-monophosphate (cGMP) regulating signalling peptides), leading to increased intracellular concentrations of the second messenger cGMP that mediates its biological effects[35]. A rise in intracellular cGMP is known to modulate a range of cellular processes, and its well-characterized intracellular effects are primarily mediated through interaction with 3 groups of target proteins: cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases and cyclic nucleotide gated ion channels[36].

Another important finding is confirmation of the increase in latency time on diosgenin administration as compared to the control vehicle treated group in the hot plate test, a model that has two types of responses: jumping and paw licking, both of which are integrated in supraspinal structures[37]. This model incorporates C and A type I and II sensitive fibers[38]. Therefore, diosgenin may be acting due to a reduction in the integration of the response in the spinal cord dorsal horn or at supraspinal levels. However, further studies are needed to confirm this hypothesis. When compared to the diosgenin-alone treated group, pretreatment with L-NAME, 7-nitroindazole, ODQ, and glibenclamide (for 10 min) prior to diosgenin treatment resulted in a dose-dependently significant decrease in latency time in the hot plate test. This suggests that the various pathways affected by L-NAME, 7-nitroindazole, ODQ, and glibenclamide may be involved in the mechanism of diosgenin's antinociceptive action (Fig. 6). The open field test results show that diosgenin (50 mg/kg p.o.) did not significantly affect motor performance, which suggests that there was no interference with motor coordination in the antinociceptive response observed due to diosgenin administration.

Conclusion

The results in the above study demonstrated maximum effect of disogenin @50 mg/kg. It inhibited the neurogenic phase and inflammatory phase of the formalin induced hyperalgesic effect. Disogenin also inhibited response integration in the spinal cord and supraspinal level. We found that pre-treatment with antagonists like L-NAME, 7-nitroindazole, ODQ and glibenclamide influenced the analgesic effect of disogenin. To nullify the motor effect of disogenin, we performed the open field test, and the results concluded that there was no significance. Based on our findings, we concluded that the NO/cGMP/KATP pathway plays an important role in disogenin's analgesic activity. However, further studies are required to confirm this effect.

Conflict of interest

Authors declare no competing interests.

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