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Basic Science

Attenuation of Mouse Somatic and Emotional Inflammatory Pain by Hydralazine Through Scavenging Acrolein and Inhibiting Neuronal Activation

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Background: Acrolein signaling is important during spinal cord injury; whether it is involved in somatic and emotional pain is not clear. Hydralazine is a potent antihypertensive drug and can scavenge acrolein efficiently.

Objective: We hypothesized that hydralazine decreases spinal level acrolein and renders analgesic effects with some side effects, which was tested in the current study.

Study design: Subcutaneous (injection of formalin was used to induce somatic and emotional pain responses. The spinal neuronal activation (FOS expression) and acrolein expression were evaluated at 2 hours after subcutaneous formalin injection. The possible side effects of hydralazine on the murine central nervous system or cardiovascular system were evaluated at one hour after hydralazine injection with open field, elevated plus maze and rotarod tests, or telemetrical measurement of mean artery blood pressure and heart rate.

Results: The subcutanous injection of formalin into the left hind paw induced significant somatic and emotional pain responses, evaluated by the biphasic spontaneous flinch/licking of the injected hind paw and interphase ultrasonic vocalizations during the one hour window after formalin injection. The spinal acrolein level was significantly increased and neurons were activated at 2 hours after formalin injection. Intraperitoneal injection of hydralazine (at 0.1, 1 or 10 mg/kg of body weight) at one hour before formalin challenging dose-dependently attenuated the formalin induced pain responses with an analgesic 50% effect dose ranging from 0.2 to 1 mg/kg of body weight. Furthermore, the neuronal activation and elevated acrolein expression were dose-dependently inhibited by hydralazine pretreatment. The side effects of intraperitoneal hydralazine on locomotion, anxiety, and motor coordination at one hour after hydralazine administration had negative results. The main side effects of hydralazine were an insignificant decrease of blood pressure and a significant increase of heart rates at high dose (10 mg/kg of body weight).

Limitations: This study is limited because the analgesic effect of hydralazine was tested on only one type of acute inflammatory pain model; however, its effect on chronic inflammatory or neuropathic pain needs to be further investigated.

Conclusions: Based on the above findings, hydralazine may find its new application of analgesia within a safe dose window (around one mg/kg of body weight) without causing severe side effects.

Key words: Inflammatory pain, acrolein, hydralazine, formalin test, ultrasonic vocalization, mouse

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The central nervous system (CNS) has a high content of polyunsaturated fatty acids which are particularly vulnerable to free radical attacks and lipid peroxidation. Aldehydic products of lipid peroxidation, 4-hydroxynonenal and acrolein, have recently become the target molecules in various neurological diseases because of their involvement in oxidative stress (1). The important role of acrolein in spinal cord injury (SCI) (2-4) and other CNS diseases (5) has been established.

Hydralazine is an antihypertension drug. It possesses an aldehyde-trapping property that is highly efficient (6). Furthermore, hydralazine significantly attenuates acrolein-mediated cell death in cultured hepatocytes (6,7), in mice (8), and in PC12 cells (9), as well as acrolein-induced and compression-induced injuries in the spinal cord ex vivo (10).

Increased oxidative stress is always observed in visceral pain (11), diabetic neuropathic pain (12), and subcutaneous formalin-injection-induced inflammatory pain (13) and leads to the increase of free radicals. Although free radical scavenging, including hydralazine, is a promising analgesic strategy, the research outcome of conventional free radical scavengers in pain remains controversial (11,14,15). Because pain is a multidimensional symptom which involves both somatic and emotional aspects, function of the acrolein-hydralazine system on these symptoms needs to be explored. Unfortunately, whether acrolein plays a role in the somatic and/or emotional aspects of pain responses remains largely unknown. Thus, we raised the hypothesis that hydralazine can alter the spinal acrolein level and render analgesic effects with some expected side effects on the cardiovascular system.

An SCI model was recently used as an experimental pain model (16); it is natural to deduce that hydralazine can induce some beneficial effects on the subsequent pain following SCI. However, the most common type of daily pain is inflammatory pain which a peripheral inflammation as well as somatic and emotional aspects. It is not clear whether the acrolein system can be changed during inflammatory pain or whether hydralazine has a benefit on the symptoms. Thus, we investigated the change on the acrolein system in an animal model of inflammatory pain, formalin pain model. Subcutaneous injection of formalin into the plantar surface of one hindpaw can induce the somatic (flinching or licking the injected hindpaw) and affective-emotional responses (USV emission) (17), which enabled us to evaluate the analgesic effect of hydralazine on somatic or emotional pain response. To test our hypothesis, intraperitoneal injection of hydralazine was performed one hour before formalin challenging, the pain responses during the one hour following formalin injections were evaluated and the spinal expression of acrolein and FOS (indicating neuronal activation) were semi-quantified at 2 hours after formalin injection. The possible side effects of hydralazine on the mice CNS or cardiovascular system were evaluated at one hour after hydralazine injection with open field , elevated plus maze (EPM) and rotarod tests or telemetrical measurement of mean artery blood pressure and heart rate. Our study raised the possibility that hydralazine could be used as analgesic agent at a safe dose window (around 1 mg/kg) without causing severe side effects.

Methods

Animal and Drugs

Male C57BL/6 mice (about 10 weeks old) were housed in a temperature-controlled environment on a 12-hour light/dark cycle with access to food and water ad libitum. Hydralazine (Sigma, St. Louis, MO; Catalog No.: M107; Batch No.: 027K4621; 5 mg) was stored at room temperature and freshly diluted in saline before use. All experimental procedures received prior approval from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, China), and the ethical guidelines to investigate experimental pain in conscious animals were followed.

Experimental Design

According to our pilot experiment, the behavioral and morphological features of mice receiving subcutaneous saline injection were similar to those of naïve mice, thus, in the current study, we only tested the naïve mice when needed. Two experiments were designed to confirm our hypothesis.

Experiment 1 aimed to elucidate the effect of intraperitoneal hydralazine pretreatment on formalin induced pain (somatic and emotional) as well as spinal neuronal activation and acrolein expression. After a 2-week acclimation period, the animals were randomly assigned to one of the following groups: 1) naïve, mice without subcutaneous formalin injection or intraperitoneal hydralazine pretreatment; 2) mice received an intraperitoneal injection of saline followed by a subcutaneous injection with 25 µl of 5% formalin one hour later (Veh group); 3) mice that receivedan intraperito-

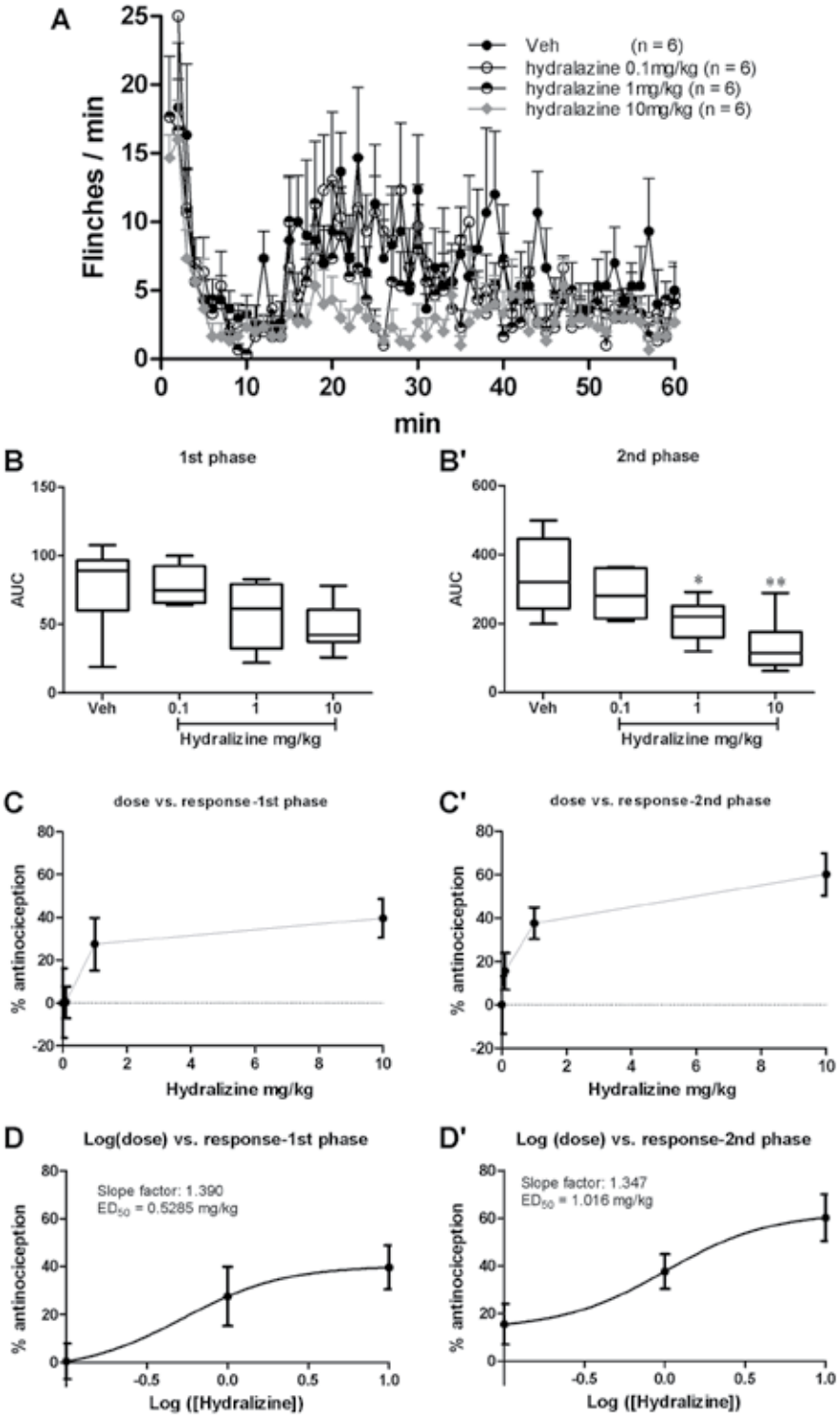

Fig. 1. *Hydralazine dose-dependently inhibited formalin induced spontaneous flinches of the injected hind paw. Spontaneous flinches during 60 minutes after subcutaneous formalin injection from different groups were shown in A. The areas under curve for different groups were calculated to perform statistical analysis on first (B) and second (B2) phases. The dose-effect or log (dose)-effect curves for hydralazine's analgesic effects were shown in C1 and D1 (first phase) or C2 and D2 (second phase).*

neal injection of 0.1 mg/kg of hydralazine followed by s subcutanous injection with 25 μl of 5% formalin one hour later (hydralazine 0.1 mg/kg group); 4) mice that received an intraperitoneal injection of 1 mg/kg of hydralazine followed by a subcutanous injection with 25 μl of 5% formalin one hour later (hydralazine 1 mg/kg group); 5) mice that received an intraperitoneal injection of 10 mg/kg of hydralazine followed by a subcutanous injection with 25 μl of 5% formalin one hour later (hydralazine 10 mg/kg group). The animals were video and audio recorded for later offline analysis during the one hour time window after formalin injection and sacrificed at 2 hours after formalin injection for morphological analysis.

Experiment 2 aimed to evaluate the possible side effects of hydralazine pretreatment on CNS and cardiovascular system functions. The animals were randomly assigned to one of the following groups: 1) mice that received an intraperitoneal injection of saline (0 mg/kg); 2) mice that received an intraperitoneal injection with 0.1 mg/kg of hydralazine (0.1 mg/kg); 3) mice that received an intraperitoneal injection with 0.1 mg/kg of hydralazine (1 mg/kg); 4) mice that received an intraperitoneal injection with 0.1 mg/kg of hydralazine (10 mg/kg). One hour after the above mentioned treatments, the animals were used for behavioral tests including open field, elevated plus maze, and rotarod tests. To observe the possible acute effects caused by hydralazine pretreatment, and to rule out possible interactions between behavioral trainings, an individual mouse was exposed to a single behavioral training only one time. To test cardiovascular system function, mice were implanted with a telemeter catheter for 7 days. On the day of testing, these mice received the above-mentioned 4 treatments, and mean artery blood pressure and heart rate were sampled for 80 minutes.

Sample Size Calculation

According to our pilot experiments, the area under curves for the first phase of the vehicle and 10 mg/kg of hydralazine-treated groups were about 90 and 40, respectively, with a standard deviation of about 30. By using these data and the online statistical power calculation tool (www.stat.ubc.ca/~rollin/stats/ssize/n2.html), the calculated statistical powers for first and second phase responses from 6 mice (per group) were 0.93 and 0.97 ($P < 0.05$). When comparing the difference between vehicle and one mg/kg hydralazine-treated groups, the sample size gives statistical powers of 0.76 and 0.71 for the first and second phase responses respectively. Thus, 6 mice were assigned to each treatment for all the behavioral experiments.

Formalin Test

The formalin test was used to induce somatic (flinching or licking the injected hind paw) and emotional responses (ultrasonic vocalization emission). After acclimating the mouse to the testing chamber for about 20 minutes, 25 μl of the 5% formalin solution (dissolved in saline) was subcutaneously injected into the plantar surface of the left hind paw using a microsyringe (Hamilton co. NV, USA) attached to a 30-gauge needle. After formalin administration, the mice were returned to the observing cage and the video and audio recordings were performed for 60 minutes, as described below.

All the behavior observations were performed in a dimly lit sound-proof room. A sound-attenuated clear Perspex testing cage (25x25x40 cm) was fitted with a reverse video camera for offline behavioral analysis. A trained observer conducted the behavioral analysis of the video recordings to determine the somatic pain responses induced by formalin. The observer was trained to provide a similar rating performance (at the 95% confidence level) for each behavior during the tests of different animals. Spontaneous flinches or lickings of the injected hindpaw were manually recorded with a stopwatch.

Ultrasonic vocalization emissions were recorded using a Mini-3 Bat Detector (Ultravox, Noldus Technology, Wageningen, The Netherlands), consisting of an audio filter and an ADAD converter and a personal computer with analysis software (Ultravox 2.0, Noldus Technology). The ultrasound detector was positioned above the cage and set to detect frequencies of 22 kHz with an amplitude filter setting of 4 to minimize background noise (18). The total number and duration of ultrasonic vibrations were recorded for every 5-minute period during the 60-minute recordings for each mouse. Environmental noise levels were standardized to minimize their influence on ultrasound recordings.

Morphological Study

Mice were anesthetized with an overdose of sodium pentobarbital (Shanghai Xinran Biotechnology CO. LTD., China) and perfused with 50 mL of 0.9% saline followed by 100 mL of 0.1 M phosphate buffer (PB, pH7.3) containing 4% paraformaldehyde. Subsequently, the fifth lumbar spinal cord segments (L5) were removed and post-fixed in the same fixative for 2 hours and

then saturated with 30% sucrose in 0.1 M PB (pH 7.4) overnight at 4°C. Transverse spinal sections (contralateral side was labeled by piercing) were cut into 25 μm thickness on a frozen microtome (Kryostat 1720; Leitz, Mannheim, Germany), collected serially into 3 dishes, each of which contained a complete series of sections.

Immunohistochemistry

Immunohistochemistry staining for FOS was performed on the first set of sections with the avidin-biotin-peroxidase complex (ABC) method. All sections from the first set were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.3) 3 times (10 minutes each), blocked with 2% of goat serum in 0.01 M PBS containing 0.3% Triton X-100 for one hour at room temperature. The sections were incubated overnight at 4°C with the rabbit primary antibody against acrolein (1:500; US Biological, US) in 0.01 M PBS containing 5% (v/v) normal donkey serum), 0.3% (v/v) TritonX-100, 0.05% (w/v) of NaN3 and 0.25% (w/v) of carrageenan (PBS-NDS, pH 7.4) at 4°C. Later, all sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) diluted in PBS-NDS for another 4 hours, and then with ABC Elite Kit (1:100; Vector) in 0.01 M PBS (pH7.4) for one hour. Between each step, the sections were completely washed with 0.01 M PBS (pH 7.4). Finally, the sections were reacted with 0.05 M of Tris–HCl buffer (pH 7.6) containing 0.04% diaminobenzidinetetrahydrochloride (DAB) (Dojin, Kumamoto, Japan) and 0.003% H2O2 for visualizing FOS.

Immunofluorescent Staining

All sections from the second set were rinsed in 0.01 M (PBS, pH 7.3) 3 times (10 minutes each), blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 for one hour at room temperature, and then used for the immunofluorescent staining. The sections were incubated overnight at 4°C with the rabbit primary antibody against acrolein (1:500; USBiological, US) diluted in 0.0 1M PBS containing 5% (v/v) of NDS, 0.3% (v/v) of TritonX-100, 0.05% (w/v) of NaN3 and 0.25% (w/v) of carrageenan at 4°C. The sections were washed 3 times with 0.01 M PBS (10 minutes each) and then incubated for 4 hours at room temperature with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1: 500; Molecular Probes, Rockford, IL) diluted in 0.01 M PBS. The specificities of the staining were tested on the sections from the other dish by omitting the primary specific antibodies. No immunoreactive products were found on the sections. Sections were air dried and cover slipped with a mixture of 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine (anti-fading agent) in 0.05 M PBS. Fluorescent images were captured under an epifluorescentmicroscope (BX60; Olympus, Tokyo, Japan). Five nonadjacent sections from the L5 segments of one mouse were selected randomly. For semi-quantification, the fluorescent intensities of acrolein-like immunoreactivities were detected on the same areas of the dorsal horn by using Image J software.

The specificities of the immunohistochemistry labeling and immunofluorescent staining were tested on the sections from the other dish by omitting the primary specific antibodies. No immunoreactive products were found on the sections.

Elevated Plus Maze Test

The Elevated Plus Maze test was done according to our previous reports (19,20) with some modifications for mice. Briefly, the Plexiglas apparatus consisted of a plus-shaped platform elevated 50 cm from the floor. Two of the opposing arms (30 cm x 5 cm) were enclosed by 25 cm high side and end walls (closed arms), whereas the other 2 arms had no walls (open arms,). Mice were placed individually into the center (neutral) zone of the maze, facing an open arm and were allowed to explore the maze for a 5-minute period. The number of open and closed arm entries and time spent in the open and closed arms were recorded. Animals were considered to be in the open or closed arms only when all 4 paws crossed out of the neutral zone. The Elevated Plus Maze Test relies on the animal's natural fear of open spaces. The percentage of time spent in open arms (open arm time percent) and percent of open arm entries (open arm entries percent) are believed to be measures of general anxiety/depression levels. Open Arm time percent was calculated by taking the time spent in the open arms and dividing it by the sum of the time spent in both open and closed arms. Open arm entries percentage was calculated by taking the number of open arm entries and dividing it by the sum of the entries into both open and closed arms (Shanghai Mobiledatum Information Technology Co., Ltd, Shanghai China).

Open Field Test

Mice were placed at the center of a cubic chamber [470mm (W)×470mm (H)×470mm (D)]. The total distance that the animal traveled in 15 minutes was measured by an automated analyzing system (Shanghai Mobiledatum Information Technology Co., Ltd). This distance was used as a parameter for the mice lo-

comotion. The percent of time spent in the center area (center time percent) is another parameter for evaluating anxiety/depression levels. All animals were acclimated to the testing room for 20 minutes before the start of the session. The test room was dimly lit with indirect white lighting, because mice are nocturnal and their natural exploratory behavior is hindered in well-lit conditions.

Rotarod Test

Motor coordination and balance were determined using a standard mouse rotarod (Shanghai Mobiledatum Information Technology Co., Ltd) which provides an accelerating rotational speed from 4 rpm at the start of the test reaching a maximum speed of 24 rpm within 120 seconds. Only one trial for each animal was performed at one hour after intraperitoneal administration of reagents (vehicle or hydralazine) by placing mice on the rotating drums (3-cm diameter) and measuring how long each animal was able to maintain its balance on the rod. The latency to fall down the rotating drums was determined automatically by a timer that was triggered by a circuit that was switched on when the mouse fell stopping the LED signal. A cut off time of 120 seconds was used for all rotarod assessments.

Blood Pressure Telemetry

Telemetric transmitters were magnetically activated 24 hours before implantation. Mice were anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively), and the left carotid artery was isolated. The telemeter catheter was inserted into the left carotid artery and advanced to reach the aortic arch, and the telemeter body (model TA11PA-C20, Data Sciences International, St. Paul, MN) was placed in a subcutaneous pocket on the right flank. One day after surgery, each animal was returned to its home cage with ad libitum food and water for the duration of the study. The telemeter signal was processed using a model RPC-1 receiver, a20-channel data-exchange matrix, APR-1 ambient pressure monitor, and a Dataquest ART 2.3 acquisition system (Data Sciences International). The system was programmed to acquire data for 10 seconds every 2 minutes and to calculate 10-minute averages of the mean artery blood pressure and heart rate). On the day of the experiment, the mice were left undisturbed for at least 5 hours while their mean artery blood pressure and heart rate were recorded. Recordings continued while the mice were treated with vehicle or hydralazine.

Data Analysis

Statistical Analysis.

The results were expressed as mean value ± standard error of the mean (SEM). In the formalin test, when comparing the somatic pain responses, data from the first phase and the second phase were considered independently; when comparing the emotional pain responses, data obtained during the first hour were pooled together. The area under curve of individual animals for formalin pain response curves (somatic and ultrasonic vocalizaitons) and cardiovascular function parameters (heart rate and mean artery blood pressure) were group pooled and One-way ANOVA with Dunnett's post hoc test was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Dose-Effect Curve and ED50 Calculation

The hydralazine dosages were transformed into a logarithm dose with Prism and the non-line fit was performed so as to build the dose-effect curve. Based on the dose-effect cure, the ED50s of hydralazine on analgesia, neuronal activation, acrolein expression, and the cardiovascular function were calculated. The reliability of ED50 calculated from a specific dose-effect curve can be evaluated by the slope factor returned by the GraphPad Prism version 5.01 for Windows (San Diego California USA, www.graphpad.com).

Results

Effect of Intraperitoneal Hydralazine on the Formalin Induced Somatic Pain Responses

The subcutanous injection of 5% formalin into the dorsal surface of the hindpaw produced biphasic pain behaviors consisting of spontaneous flinching and licking/biting the injected paw: the first transient phase lasted for the first 10 minutes post injection and was followed by the second prolonged phase beginning from 10 minutes post injection. There was no difference in the time course of the formalin-induced pain behaviors between groups with and without intraperitoneal hydralazine pretreatment (Fig. 1A and Fig. 2A). The flinching, licking, or biting behaviors are always used in a pain scoring system to evaluate the spontaneous pain responses. In order to double check the dose-analgesic effect of hydralazine on the formalin-induced somatic pain responses, we analyzed the spontaneous flinching or licking behaviors, respectively.

Spontaneous Flinches

An obvious biphasic flinch response can be induced by the subcutanous injection of formalin (Fig. 1A). Pretreatment with intraperitoneal hydralazine significantly affected the second but not the first phase flinches. (1) First phase. There was no group difference in the first phase flinches [Fig. 1A and 1B; one way ANOVA (between-subject factor: treatment) F(3, 23) = 2.859, P = 0.0628]. Dunnett's post hoc test also revealed no group difference within these 4 groups. The effect of hydralazine on the first phase flinches was calculated based on the log (dose) vs response curve (Fig. 1D) from the dose vs response curve (Fig. 1C). The ED50 of hydralazine on the first phase flinches was 0.5285 mg/kg with a slope factor of 1.390 suggesting that such a regime for dosage selection was relatively feasible. (2) Second phase. There was a significant a group difference in the second phase flinches [Fig. 1A and 1B'; one way ANOVA (between-subject factor: treatment) F (3, 23) = 6.938, *P* = 0.0022]. Dunnett's post hoc test also revealed group difference between 1mg/kg ($P < 0.05$) or 10 mg/kg ($P < 0.01$) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups ($P > 0.05$). The effect of hydralazine on the second phase flinches was calculated based on the log (dose) vs response curve (Fig. 1D') from the dose vs response curve (Fig. 1C'). The ED50 of hydralazine on the second phase flinches was 1.0160 mg/kg with a slope factor of 1.347 suggesting that such a regime for dosage selection was relatively reliable.

Spontaneous Lickings.

An obvious biphasic licking response with a lower second peak can be induced by the subcutanous injection of formalin (Fig. 2A). Pretreatment with intraperitoneal hydralazine significantly affected both phase lickings. (1) First phase. There was a significant group difference in the first phase lickings [Fig. 2A and 2B; one way ANOVA (between-subject factor: treatment) F (3, 23) = 5.765, *P* = 0.0052].Dunnett's post hoc test also revealed significant group difference between 1mg/kg ($P < 0.01$) or 10 mg/kg ($P < 0.01$) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups ($P > 0.05$). The effect of hydralazine on the first phase flinches was calculated based on the log (dose) vs response curve (Fig. 2D) from the dose vs response curve (Fig. 2C). The ED50 of hydralazine on the first phase flinches was 0.3656 mg/kg with a slope factor of 1.578 suggesting such a regime for dosage selection was relatively feasible. (2) Second phase. There was a significant group difference in the second phase flinches [Fig. 2A and 2B'; one way ANOVA (between-subject factor: treatment) F (3, 23) = 5.493, *P* = 0.0064]. Dunnett's post hoc test also revealed significant group difference between 1mg/kg ($P < 0.05$) or 10 mg/kg ($P < 0.01$) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups ($P > 0.05$). The effect of hydralazine on the second phase lickings was calculated based on the log (dose) versus response curve (Fig. 2D') from the dose versus response curve (Fig. 2C'). The ED50 of hydralazine on the second phase flinches was 0.4047 mg/kg and with a slope factor of 1.509 suggesting such a regime for dosage selection was relatively reliable.

Although there were slight differences in the ED50 of hydralazine on the spontaneous flinches and lickings, these data collectively suggested that hydralazine can dose-dependently inhibit somatic pain induced by subcutanous injection of formalin into one mouse hind paw. Why hydralazine showed significant dose-dependent inhibition on the formalin-induced first phase lickings but not flinches needs to be further studied.

Effect of Intraperitoneal Hydralazine on the Formalin Induced Emotional Pain Responses Revealed by Ultrasonic Vocalizations.

According to a previous report, ultrasonic vocalizations during the formalin test can be used as a measure of the affective dimension of pain in rat (17). Sound with a frequency higher than 20 Hz is ultrasonic vocalizations, thus we filtered ultrasonic vocalizations at 22 Hz and made the statistical analysis. Consistent with the previous study made on rats, subcutaneous formalin injection into the mouse hind paw triggered ultrasonic vibrations with the peak at 10 to 20 minutes (Fig. 3A, interphase) after injection. There was a significant group difference in the ultrasonic vibrations during one hour after formalin injection [Fig. 3A and 3B; one way ANOVA (between-subject factor: treatment) F (3, 23) = 7.283, *P* = 0.0017]. Dunnett's post hoc test also revealed significant group difference between 1 mg/kg ($P < 0.01$) or 10 mg/kg ($P < 0.01$) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups ($P > 0.05$). The effect of hydralazine on the ultrasonic vocalizations was calculated based on the log (dose) vs response curve (Fig. 3D) from the dose versus response curve (Fig. 3C). The ED50 of hydralazine on the ultrasonic vocalizations was 0.4606 mg/kg with a slope factor of 1.441 suggesting such a regime for dosage selection was relatively feasible.

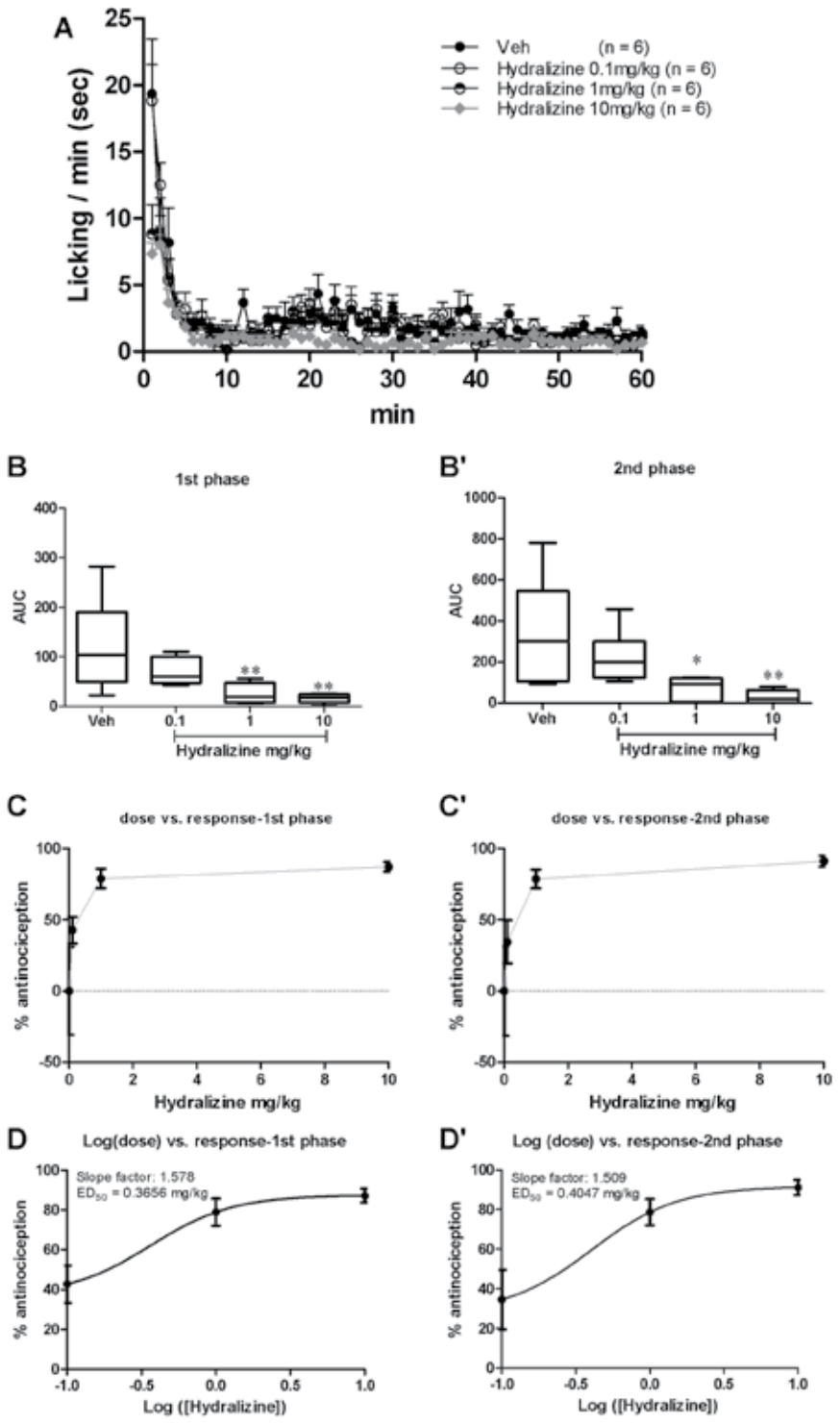

Fig. 2. *Hydralazine dose-dependently inhibited formalin-induced spontaneous lickings of the injected hind paw. Spontaneous lickings during 60 minutes after subcutaneous formalin injection from different groups were shown in A. The area under curves for different groups were calculated to perform statistical analysis on first (B) and second (B') phases. The dose-effect or log (dose)-effect curves for hydralazine's analgesic effects were shown in C and D (first phase) or C' and D' (second phase).*

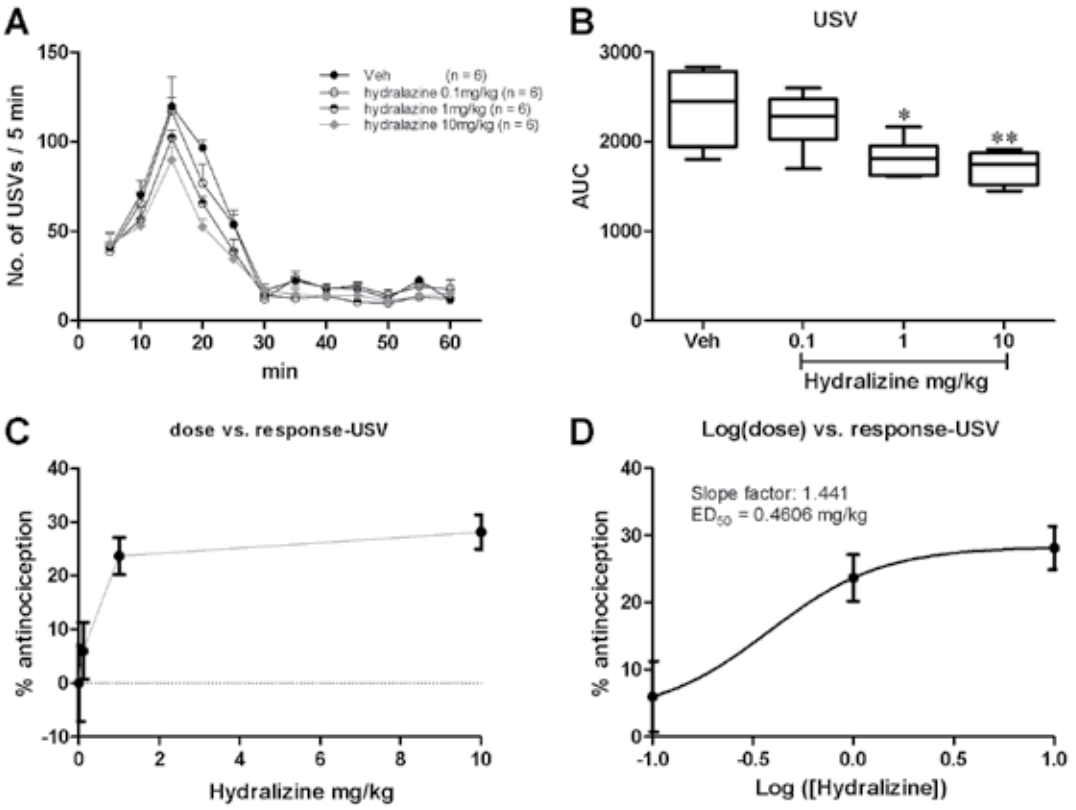

Fig. 3. *Hydralazine dose-dependently inhibited formalin induced ultrasonic vocalizations especially during the interphase (10 to 25 minutes after formalin injection). Ultrasonic vocalization curves from different groups were shown in A. The area under curves for different groups were calculated to perform statistical analysis (B). The dose-effect or log (dose)-effect curves for hydralazine's analgesic effects were shown in C and D.*

Effect of Intraperitoneal Hydralazine on the Spinal Acrolein Expression and Neuronal Activation

To test our hypothesis that intraperitoneal pretreatment with hydralazine can reverse the formalin-induced spinal acrolein increase, we first observed the spinal acrolein levels of control and formalin-injection groups. Subcutaneous injection of formalin induced a significant increase of spinal acrolein (Fig. 4A, Averaged fluorescent intensity, naïve 27.56 ± 0.64% versus vehicle 33.87 ± 0.48%, paired t-test, P = 0.0007) at 2 hours after subcutaneous injection. There was a significant group difference in the spinal acrolein levels at 2 hours after formalin injection [Fig. 4A and 4C; one way ANOVA (between-subject factor: treatment) F (3, 23) = 5.493, P = 0.0064]. Dunnett's post hoc test also revealed significant group difference between 1mg/kg (P < 0.05) or 10 mg/kg (P < 0.01) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups (P > 0.05). The effect of hydralazine on the spinal acrolein levels were calculated based on the log (dose) versus response curve (Fig. 4E') from the dose vs response curve (Fig. 4D'). The ED50 of hydralazine on the spinal acrolein levels was 0.2397 mg/kg with a slope factor of 2.092 suggesting such a regime for dosage selection was relatively feasible.

According to the previous studies from other (21) and our (22) groups, subcutaneous injection of formalin significantly increased neuronal activation indicated by the nuclei expression of FOS and such neuronal ac-

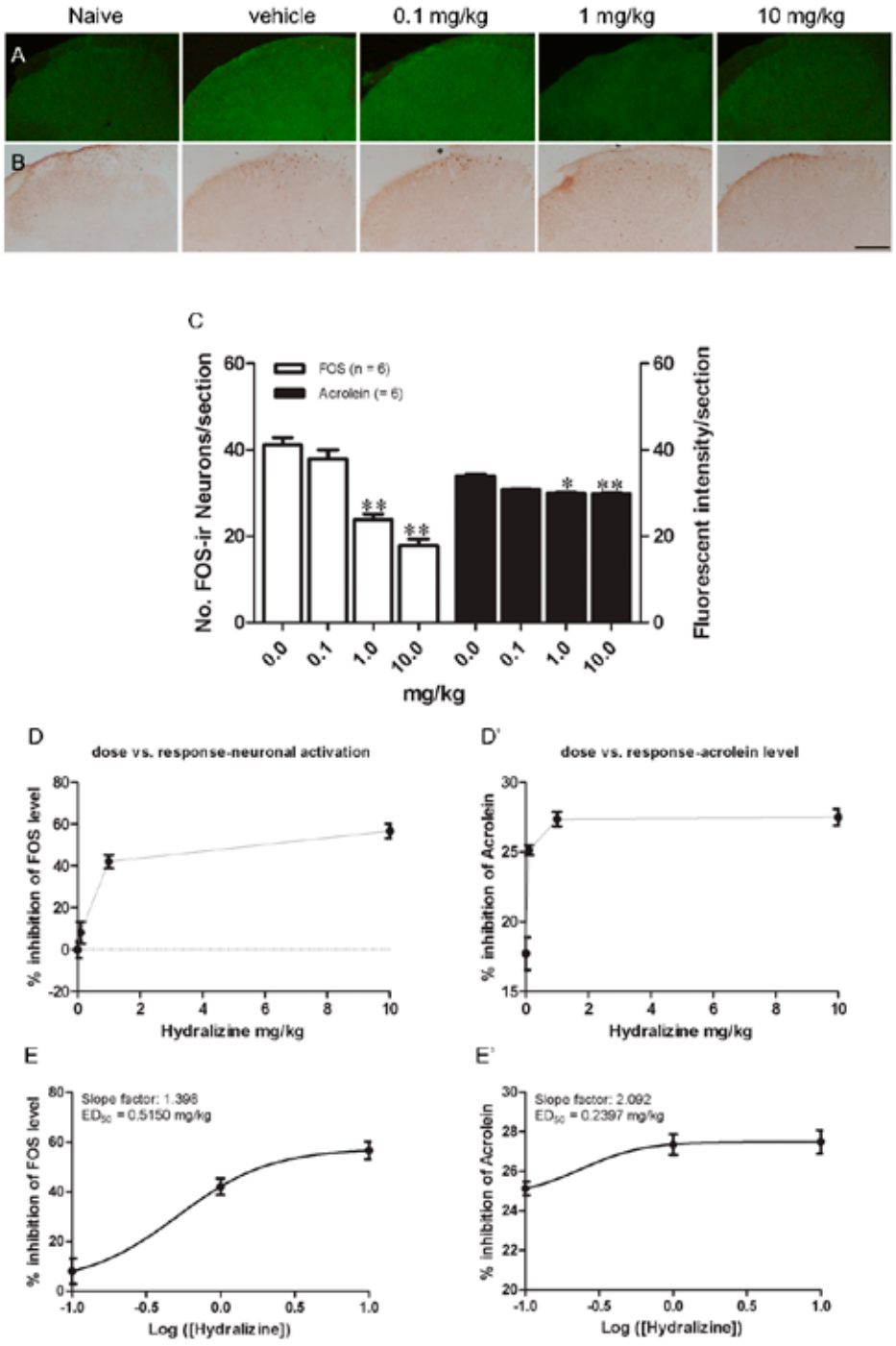

Fig. 4. *Hydralazine dose-dependently inhibited spinal acrolein expression as well as neuronal activation. Representative immunofluorescent labeling of acrolein (A) or immunohistochemical staining for FOS (B) in the left spinal cord from naïve, vehicle, 0.1mg/kg, 1mg/kg and 10mg/kg hydralazine treated group. The pooled data were showed in C and the dose-effect or log (dose)-effect graph for acrolein level or FOS-ir neurons were shown in D,E and D', E' respectively. Scale bar = 100 μm.*

tivation reaches the peak at 2 hours after formalin injection in both the rat (22) and the mouse (21). In the current study, we evaluated the FOS-immunoreactive neurons within the superficial layers of the spinal dorsal horn at 2 hours after subcutaneous formalin injection. Photomicrographs of FOS- immunoreactive neurons in the left (ipsilateral) L5 spinal dorsal horn in the naïve, vehicle, 0.1, 1 and 10 mg/kg hydralazine treated groups were shown in Fig. 4B. Subcutaneous saline injection into one hind paw can induce rare FOS- immunoreactive neurons within the ipsilateral spinal dorsal horn, which was similar to the case of the naïve mouse (Fig. 4B). The total number of FOS- immunoreactive neurons per section in these groups was shown in Fig 4C. FOS-ir neurons were rarely observed in the right (contralateral) spinal dorsal horn in the subcutaneous saline injection group or the naïve group (data not shown). In contrast, numerous FOS- immunoreactive neurons were detected in the left (ipsilateral) dorsal horn, predominantly in the superficial laminae of the L5 spinal dorsal horn of vehicle treated mice (Fig. 4B and 4C, number of FOS-ir neurons/section, naïve 0 ± 0 vs vehicle 41.17 ± 1.17, $P < 0.01$). There was a significant group difference in the spinal FOS-ir levels at 2 hours after formalin injection [Fig. 4B and 4C; one way ANOVA (between-subject factor: treatment) F (3, 23) = 5.765, $P = 0.0052$]. Dunnett's post hoc test also revealed a significant group difference between 1 mg/kg ($P < 0.01$) or 10 mg/kg ($P < 0.01$) with vehicle treatments. There was no significant difference between 0.1 mg/kg and vehicle treatment groups ($P > 0.05$). The effect of hydralazine on the number of spinal FOS-ir neurons was calculated based on the log (dose) vs response curve (Fig. 4E) from the dose vs response curve (Fig. 4D). The ED50 of hydralazine on the spinal acrolein levels was 0.5150 mg/kg with a slope factor of 1.398, suggesting that such a regime for dosage selection was relatively feasible.

Acute Effect of Intraperitoneal Hydralazine on Some CNS Functions.

The pain behaviors are accompanied with increased anxiety and reduced locomotion (23).On the other hand, emotional change such as depression can affect the formalin-induced inflammatory pain responses (24,25). The analgesic effects of hydralazine in the current study might be caused by the alteration of the mice's depressive status or locomotion. Furthermore, for an ideal analgesic agent, fewer side effects are always expected; thus we tested the possible side effects at one hour after intraperitoneal hydralazine treatment on naïve mice for locomotion, anxiety/depression, and motor coordination by using open field, elevated plus maze and rotarod tests, respectively. There was no significant group difference in the locomotion revealed by the total distance traveled during the 15-minute recording time in open field [Fig. 5A; one way ANOVA (between-subject factor: treatment) F (3, 23) = 0.3696, $P = 0.7758$]. Dunnett's post hoc test also revealed no significant group difference between 0.1 mg/kg ($P > 0.05$), 1 mg/kg ($P > 0.05$), or 10 mg/kg ($P > 0.05$) with vehicle treatments. In the open field test, there was no significant group difference in the per-

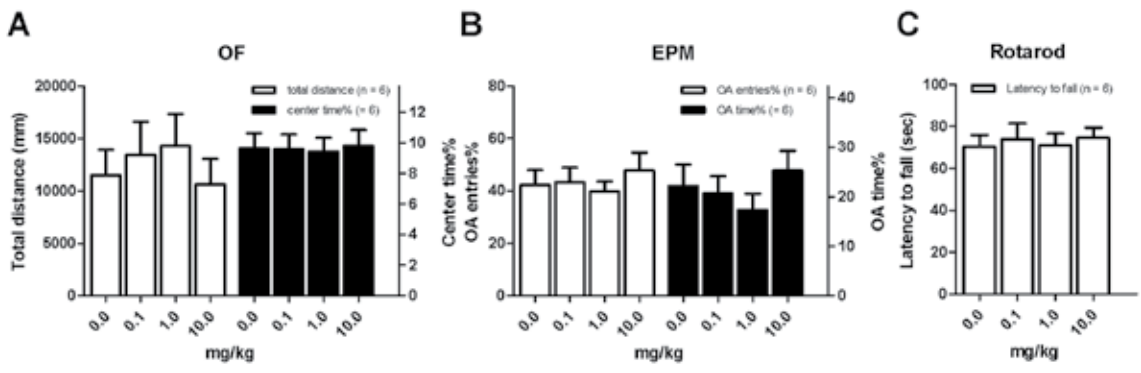

Fig. 5. *Hydralazine did not affect the animals' locomotion, anxiety level, or motor coordination. Total distance traveled and center time% in open field (A), open arm entries percenatge and open arm A time percentage in elevated plus maze (B) and latency to fall in rotatod test (C) were used to detect the locomotion, anxiety/depression and motor coordination, respectively.*

centage of center time [Fig. 5A; one way ANOVA (between-subject factor: treatment) F(3, 23) = 0.02568, P = 0.9943], which indicated no difference in their anxiety/depression status. An insignificant difference in anxiety/depression was indicated by the open arm entries percentage [Fig. 5A; one way ANOVA (between-subject factor: treatment) F (3, 23) = 0.3482, *P* = 0.7908] and open arm time percentage [Fig. 5B; one way ANOVA (between-subject factor: treatment) F (3, 23) = 0.7394, *P* = 0.5409]. Furthermore, intraperitoneal hydralazine pretreatment did not alter motor coordination [Fig. 5C; one way ANOVA (between-subject factor: treatment) F (3, 23) = 0.1214, *P* = 0.9464].

Acute Effect of Intraperitoneal Hydralazine on the Cardiovascular System Functions

The major concern of hydralazine pretreatment in the analgesic field is its effects on cardiovascular system functions. To detect how intraperitoneal hydralazine affected cardiovascular system functions, mean artery blood flow (Fig. 6A) and heart rate (Fig. 6A') at 10 min interval from 20 minutes before to 60 minutes after intraperitoneal hydralazine pretreatment were retrieved upon intraperitoneal treatment with vehicle or hydralazine at different doses. There was no significant group difference in the mean artery blood flow [Fig. 6B; one way ANOVA (between-subject factor: treatment) F (3, 23) = 1.154, *P* = 0.3518]. Dunnett's post hoc test also revealed no significant group difference between 0.1 mg/kg (*P* > 0.05), 1 mg/kg (*P* > 0.05) or 10 mg/kg (*P* > 0.05) with vehicle treatments. However, there was a significant group difference in the heart rate [Fig. 6B'; one way ANOVA (between-subject factor: treatment) F (3, 23) = 5.578, *P* = 0.0060]. Such a difference was caused by the group difference between 10 mg/kg (*P* < 0.01) and vehicle treatments. Taken together, pretreatment with intraperitoneal hydralazine at 0.1, 1, or 10 mg/kg caused no severe cardiovascular system disorders, except for the increased heart rate (at 10 mg/kg, **P** < 0.01 vs vehicle). Such a heart rate increase occurred at 10 minutes after hydralazine treatment and lasted during the 60-minute observing window (Fig. 6A').

Discussion

To our knowledge, this is the first report showing a benefit of hydralazine on the somatic and emotional aspects of inflammatory pain induced by subcutanous injection of formalin into the hind paw. Although acrolein has a well-established role in SCI and hydralazine may ameliorate the SCI via scavenging spinal acrolein, their roles in nociception and analgesia remain largely unknown. Our data support the view that subcutanous injection of formalin increases the spinal acrolein production as well as neuronal activation. Hydralazine pretreatment efficiently scavenges spinal acrolein and neuronal activation and thus inhibits nociceptive transmission (analgesia).

Our results further suggest that intraperitoneal injection of hydralazine can inhibit both somatic and emotional pain responses induced by subcutaneous formalin challenging and the analgesic dosage was within a safe range without causing obvious cardiovascular system disturbances. Thus, hydralazine may serve as a therapeutic strategy for pain control besides its antihypertensive effect (26).

Hydralazine May Find its New Application in the Antinociception Field.

The analgesic effect of hydralazine favored both somatic and emotional pain responses caused by subcutaneous formalin injection, which suggested that the functional sites are located centrally and peripherally. Although the formalin test is one of the most commonly used biphasic pain models in screening some potential analgesics and therapeutic strategies, its underlying mechanisms remain largely controversial (17,27,28) and the evaluating parameters should be carefully conducted so as to get reliable data (29). Pain scientists seem to accept the concept that both phases belong to the somatic responses of the formalin challenging, although such somatic responses may be controlled by the descending facilitation system (28). On the other hand, the ultrasonic vocalizations are an aversive audible response to the formalin challenging (17,18). Hydralazine pretreatment (intraperitoneal.) caused dose-dependent analgesia on the emotional (ultrasonic vocalizations) as well as somatic pain responses (flinches and lickings), suggesting that it may have potential as an analgesic as well as its use as a antihypertensive agent.

Hydralazine Favors the Second More than the First Phase Formalin Pain Responses Via the Spinal/Supra-Spinal Mechanisms

Our current study suggested that hydralazine pretreatment attenuates second phase formalin pain response more than that of the first phase. It is highly possible that this effect is mediated by scavenging of the spinal acrolein. According to the conventional view, the first phase formalin response is mostly due to the peripheral sensitization of the peripheral nociceptor

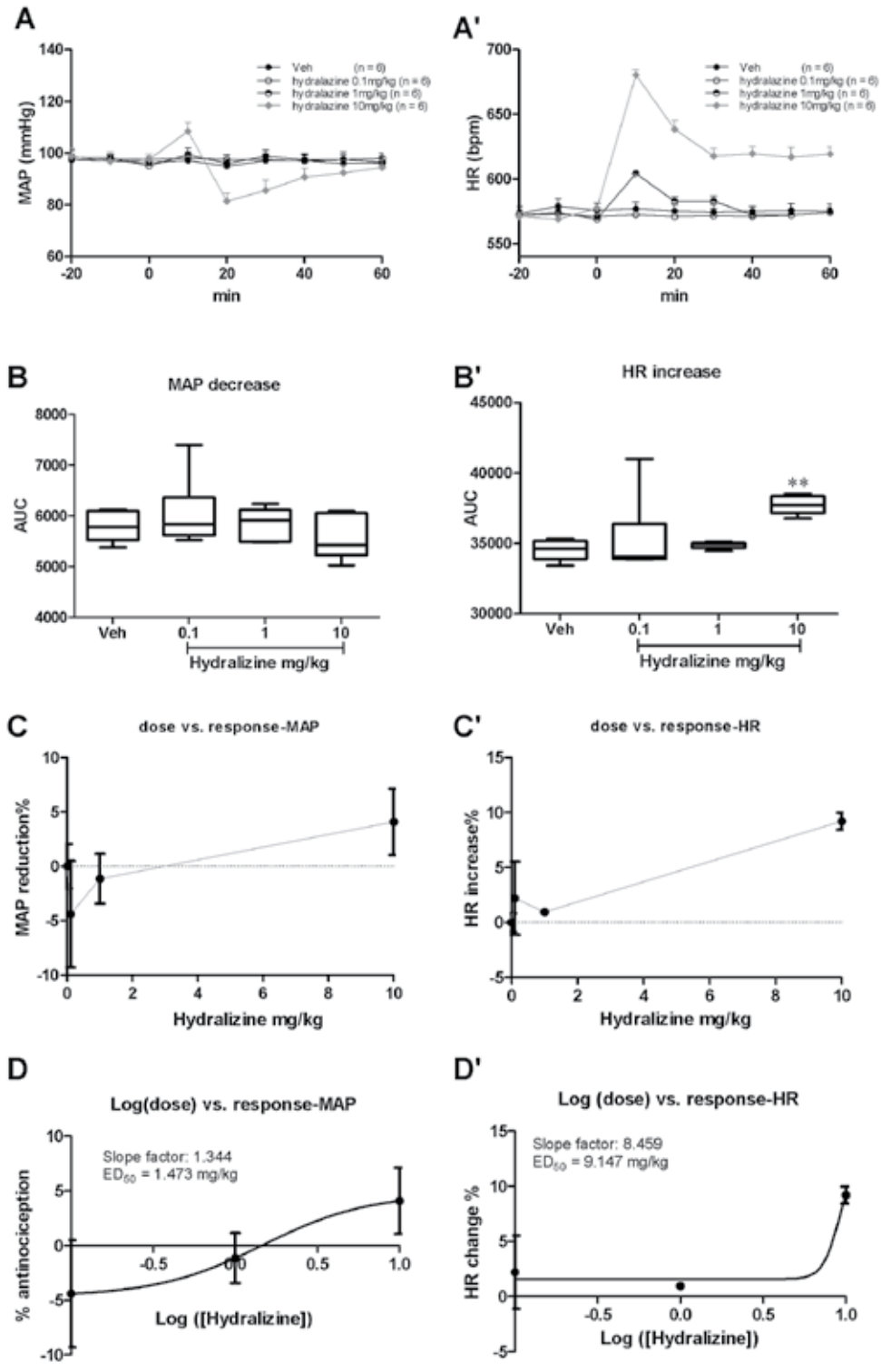

Fig. 6. *Effect of intraperitoneal hydralazine on the mice median art flow (A, B and C) and heart rate (A', B' and C').*

via direct activation of the transient receptor potential ankyrin (TRPA)-1 receptors (30); while the second phase formalin response is associated with stimulation of TRPA1 (31) and also with the development of an inflammatory response triggered by mediators such as interleukin (IL)-1b, IL-6, IL-8, tumor-necrosis factor (TNF)-α (32) and NO (33).The second phase formalin response is believed to be the consequence of central sensitization that involves spinal cord neurons (27,28,30) or primary sensory neurons (34). Based on a previous study focusing on the effects of intraperitoneal injected hydralazine, at a dose over 1 mg/kg renders effect mainly at the spinal level (35). In the current study, intraperitoneal pretreatment with hydralazine may also scavenge spinal acrolein and thus attenuate the second phase pain response that is mainly based on spinal mechanisms.

The licking behaviors were used to evaluate the formalin-induced inflammatory pain responses in mice at the very beginning (36). Later-on, other behaviors including biting, flinching etc were integrated into a scoring system with the hope of getting a relatively unbiased evaluation of formalin pain(37, 38). However, the mice's responses to subcutaneous formalin are slightly different in case of flinches and licking, with the former one lasting longer (39). In our study, this difference was clearly shown in that the second peak of licking was far less than the first one (Fig. 2A), while the second phase of flinches was similar to the first one and lasted to about 50 minutes after formalin injection (Fig. 1A). However, hydralazine pretreatment dose-dependently inhibited the second phase responses for both flinches and lickings as well as the first phase for lickings. These detailed observations confirmed the dose-dependent analgesic effect of hydralazine on the somatic aspect of inflammatory pain.

Mild Increase of Acrolein May Activate the Spinal Cord Neurons and Enhance Nociceptive Transmission

In our study, the subcutaneous formalin injection caused a mild increase of spinal acrolein which may activate the spinal neurons instead of causing neuronal damage or apoptosis. At as early as 4 h after SCI, spinal acrolein production increased drastically to more than two-fold that of the control (3). Due to the strong toxicity of the high level of acrolein, the spinal neurons were damaged and killed finally. However, if the oxidative stress was not so strong, and the free radicals were not drastically increased, the destination of the cultured (40) or in vivo spinal neurons (41) can be activated instead of killed. At 2 hours after subcutaneous formalin challenging, spinal acrolein production was about 122.9% of that of the control, such an increase was mild and it's feasible to detect neuronal activation. We, indeed, detected a significant neuronal activation as observed in the previous studies (21-23). Such increased acrolein levels and neuronal activation are consistent with the formalin-induced pain responses.

Our investigation further revealed that formalin-induced acrolein overproduction partly accounts for the neuronal activation and nociceptive responses. Upon pretreatment with hydralazine at different dosages, spinal acrolein was scavenged (7). Such a change in acrolein levels was accompanied with inhibited neuronal activation and decreased pain responses. Our findings suggested that hydralazine pretreatment can inhibit neuronal activation via scavenging spinal acrolein. On the other hand, our data also suggested that acrolein medicated oxidative stress responses contribute partly to the subcutaneous formalin-induced neuronal activation as well as inflammatory pain responses.

Since the hydralazine dosages used in the current study were without severe side effects, we raised the point that hydralazine can serve as an adjunctive therapy with other analgesic agents to control pain.

Discrepancy of the ED50 for Different Pain Parameters, Neuronal Activation, Acrolein Level as Well as Cardiovascular System Functions

ED50 is an indicator to compare the efficacy of a target agent (42). However, in the current study, the ED50 of hydralazine calculated for different parameters was slightly different. This might be due to the Hill slopes of different parameters used in the current study. For a neuropharmacology study, an ideal dose-effect curve is critical to get the ED50 value. The slope factor describes the steepness of a dose-effect curve. In most situations, there is no way to interpret the value of the slope factor in terms of chemistry or biology. However, if the slope factor is far from 1.0, then the binding does not follow the law of mass action with a single site. Thus, the calculated ED50 from the dose-effect curve may be biased. In the current study, the slope factors for flinches, lickings, ultrasonic vocalizations and FOS expression were around 1.3, 1.5, 1.4 and 1.4 respectively. These values were still within the tolerable range. And this was why the ED50's for these parameters are within a limited range (0.4-1.6 mg/

kg). However, the slope factors for acrolein expression and heart rate were 2.1 and 8.459, respectively. These values were far beyond the tolerable range. Thus the ED50's for these two parameters varied so much, 0.24 and 9.147 for acrolein expression and heart rate, respectively.

In summary, our present study offers experimental supoort that intraperitoneal hydralazine pretreatment can inhibit subcutaneous formalin-injection-induced pain responses as well as the spinal increase of acrolein and neuronal activation in the spine. With the dosages of hydralazine used in the current study, no severe side effects were observed. Thus, hydralazine may scavenge the formalin-induced acrolein over-production and the downstream neuronal activation, leading to an analgesic effect. Further resaerch is needed to investigate whether hydralazine may serve as a therapeutic option for pain control as well as its antihypertensive effect.

Competing Interest Statement and Acknowledgement.

Drs. Lu Bai, Wen Wang and Yu-Lin Dong contribued equally to this manuscript as first authors. All authors have completed the Unified Competing Interest form and declare there was no financial relationship with any organization that might have an interest in the submitted work during the previous 3 years, and there are no other relationships or activities that could appear to have influenced the submitted work.

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