Heartworm extract induces relaxation of isolated rat thoracic aorta

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ABSTRACT. Isolated rat thoracic aortic strips undergoing noradrenaline-induced contraction were treated with an adult heartworm (HW) crude extract and then examined for isometric changes in tension. HW extract caused relaxation of endothelium-intact strips, but not endothelium-denuded strips. This effect was inhibited by treatment with N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and could be reversed by additional treatment with L-arginine. However, HW extract at a high concentration caused slight relaxation of endothelium-denuded strips, and relaxation persisted after L-NAME treatment in endothelium intact-strips. These data suggested that the relaxation induced by HW extract was mainly endothelium-dependent, nitric oxide-mediated, but in part, also endothelium-independent. In addition, a bioassay using isolated rat thoracic aortas may be a useful tool for investigating vasoactive substances in the HW extract.

KEY WORDS: *Dirofilaria immitis*, heartworm extract, rat aorta, relaxation

Canine cardiopulmonary dirofilariasis, which is caused by the filarial nematode *Dirofilaria immitis* (heartworm, HW), is a serious and potentially life-threatening disease [20]. Adult HWs reside for years in pulmonary arteries close to vascular endothelial cells. During this time, HW body components interact with vascular endothelial cells and smooth muscle cells. This interaction alters the capacity of the vascular wall to contract and relax, and thereby contributes to the pathogenic processes that lead to canine cardiopulmonary disease [4, 6, 17, 22].

Our previous study showed that HWs contain at least two vasoactive substances; one causes vasoconstriction via direct action on the vascular smooth muscle, and another causes vasodilation indirectly by releasing nitric oxide (NO) from vascular endothelium [10]. It is possible that these vasoactive substances released into the host circulation may play a role in the pathogenesis of cardiopulmonary dirofilariasis. Thus, their purification and identification may be important to elucidate disease pathogenesis.

Our previous study assayed HW extract activity using isolated canine abdominal aortas, which required preparation of blood vessel specimens from euthanized dogs for each experiment. The use of such an approach, however, is difficult from an ethical viewpoint. In order to purify and identify vasoactive substances from HW extracts in a bioassay system, a continuous supply of blood vessel specimens is needed.

A few factors produced by adult HWs depress agonist-induced, endothelium-dependent relaxations of isolated rat aorta and increase acetylcholine-induced contractions of the rat trachea *in vitro* [1, 5, 8, 11]. However, relaxation of the rat aorta by substances contained in HWs has not been reported. The purpose of this study was to examine whether HW extracts relax isolated rat thoracic aortas *in vitro*, and in doing so, to confirm that a bioassay using isolated rat thoracic aorta may be a useful tool for investigating vasoactive substances in HW extracts.

This study complied with the Gifu University guidelines for animal experimentation. HW extract was prepared as reported previously [9]. Briefly, 10 adult female uninjured HWs were obtained from HW-infected dogs, washed several times with physiological saline (0.9% sterilized NaCl solution), cut into small fragments using scissors and a mechanical mixer, and then sonicated 10 sec ×10 times in 20 m*l* saline containing 1% Tween 80 (polyoxyethylen sorbitan monooleate, Sigma-Aldrich Corp.,

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St. Louis, MO, U.S.A.) and 1 mM EDTA (disodium dihydrogen ethylenediaminetetraacetate dehydrate, Nacalai Tesque, Inc., Kyoto, Japan). The worm suspension was centrifuged at 10,000 g for 40 min, and the supernatant was collected. All steps were conducted at 4°C. Two ml of this solution was equivalent to the volume extracted from 1 HW (low concentration [LC] extract). Five-fold concentrated extract (high concentration [HC] extract), of which 2 ml was equivalent to the volume extracted from 5 HWs, was prepared in the same way. Both LC and HC extracts (0.2 ml each) were stored at −80°C until use.

Nineteen male Wistar rats, aged 8–26 weeks (Japan SLC, Inc., Hamamatsu, Japan), were used to prepare blood vessel specimens for HW extract bioassays. Rats were anesthetized with ether and euthanized by exsanguination. The thoracic aorta (approximately 30 mm long) was immediately removed and placed in Tyrode's solution composed of: (mM) NaCl, 136.9; KCl, 2.68; CaCl₂, 1.8; MgCl₂, 2.1; NaH₂PO₄, 0.41; NaHCO₃, 11.9; and glucose, 5.55. Connective and adipose tissues were removed, and several helical strips (approximately 4 mm wide and 25 mm long) were dissected from the isolated aorta with care so as to not separate the endothelium from the luminal surface. When aortic strips without endothelium were required, their inner surface was gently rubbed with wet filter paper. Assay tissues were mounted in a 5-ml organ bath by connecting one cut end to a stationary holder in the bath with silk thread and connecting the other end to a force transducer. The bath was filled with Tyrode's solution, maintained at 37°C and aerated continuously. The tissues were loaded with an initial tension of 0.5 g and equilibrated for 80 min, during which the bath solution was changed several times. Changes in isometric tension of the tissues were recorded by a force-displacement transducer (model T7-30-240, A&D Co., Ltd., Tokyo, Japan) coupled with a strain DC amplifier (AS2102, NEC San-ei Co., Ltd., Nagoya, Japan), the output being displayed on a polygraph (model Unicorder U-228, PANTOS Co., Ltd., Nagoya, Japan).

Aortic strip relaxation and contraction upon exposure to HW extract were examined on assay tissues precontracted with 0.1 μM noradrenaline (NA; Sigma-Aldrich Corp.). HW extract (50 μl) was injected into the bath solution after NA-induced contraction reached plateau levels and was allowed to act on the assay tissue for 6 to 18 min. The added substances, such as NA and HW extract, were then removed by replacing the bath solution with fresh Tyrode's solution. Before assaying HW extract, functional integrity of endothelium was checked by carbamylcholine chloride (CCh; Wako Pure Chemical Industries Ltd., Osaka, Japan), which is known to relax arterial smooth muscle in an endothelium-dependent manner [19]. We designated specimens that had more than 50% relaxation of the NA-induced contraction by CCh (100 μM) as being aortic strips with functional endothelium. Specimens that were not relaxed by CCh (100 μM) were designated as aortic strips without functional endothelium, although the possibility that some endothelial cells remained on the strip surface was not excluded. The relaxation in response to HW extract or CCh was expressed as a percentage of the NA-induced contraction. Results are expressed as means ± SD. Statistical evaluation of difference between means was performed with a Student's unpaired t-test and ANOVA followed by the Tukey-Kramer method for pairwise comparisons when more than two groups were compared. A P value less than 0.05 was considered significant.

The effect of HW extracts on NA-precontracted aortic strips was examined after endothelium integrity was confirmed by an ability of CCh (100 μM) to induce relaxation (70.5 ± 17.1%, n=10) (Fig. 1A *left* and 1B). LC extract (50 μl) caused relaxation of the aortic strip. Relaxation started immediately after injection of the extract into the bath solution, reached a peak in 6 to 8 min and then persisted (Fig. 1A *middle*). The size of the relaxation was 33.0 ± 16.3% (n=8) (Fig. 1B). HC extract (50 μl) elicited a similar effect to that of the LC extract (35.4 ± 11.0%, n=5, not significant compared with the size of the relaxation induced by LC extract) (Fig. 1A *right* and 1B). In endothelium-denuded aortic strips, as judged by the inability of CCh (100 μM) to induce relaxation (0 ± 0%, n=8) (Fig. 2A *left* and 2B), LC extract-induced relaxation was significantly abolished (Fig. 2A *middle*) (1.0 ± 1.5%, n=5 vs. 33.0 ± 16.3%, n=8 endothelium-intact strips, P<0.01, Fig. 2B). In contrast, HC extract-induced relaxation of endothelium-denuded strips was completely abolished in only 3 out of 7 strips, with 4 out of 7 strips still showing slight relaxation (Fig. 2A *right*). The degree of relaxation in these 7 strips was 5.4 ± 6.5% (not significant compared to relaxation induced by LC extract, P<0.01 compared with the value in endothelium-intact strips, 35.4 ± 11.0%, n=5, Fig. 2B). Thus, the relaxation induced by HW extract was mostly endothelium-dependent, but in part, especially at high concentrations, also endothelium-independent.

The activity of HW extracts to relax aortic strips was mostly endothelium-dependent, and thus, it was further characterized with agents that affect NO-mediated signal transduction [14, 15]. Treatment with 300 μM N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Wako Pure Chemical Industries Ltd.), an NO synthase inhibitor, to the bath solution 30 min before the addition of NA significantly reduced the relaxation due to LC extract treatment in 5 out of 5 endothelium-intact strips (27.2 ± 11.1% reduced to 3.3 ± 4.0%, P<0.01) (Fig. 3A *left*, *middle* and 3B). This reduction upon L-NAME treatment was significantly attenuated by addition of 30 μM L-arginine (Wako Pure Chemical Industries Ltd.), a substrate for NO synthesis, to the bath solution 30 min before addition of NA (20.6 ± 10.2% in 3 out of 3 strips, P<0.05 when compared to the value produced by LC extract plus L-NAME treatment) (Fig. 3A *right* and 3B). Relaxation induced by LC extract and by addition of L-arginine was not significantly different. In contrast, relaxation due to HC extract treatment decreased from 37.2 ± 9.2% to 17.0 ± 17.2% after treatment with 300 μM L-NAME, but the difference did not reach statistical significance (P=0.1185) (Fig. 4A *left*, *middle* and 4B). Moreover, additional treatment with 30 μM L-arginine increased the degree of relaxation to 35.1 ± 20.5% in 3 out of 3 strips, but the difference did not reach statistical significance (Fig. 4A *right* and 4B).

Structural analogues of L-arginine, such as L-NAME, are the most specific inhibitors of NO synthase [7, 15]. Inhibition by these analogues is specifically reversed by L-arginine [7, 15]. Complete inhibition of LC extract-induced relaxation by L-NAME and restoration of the effect with L-arginine, suggested that the response of the thoracic aorta is mediated entirely by NO. However, HC extract caused slight relaxation in 4 out of 7 endothelium-denuded strips, and relaxation persisted after L-NAME treatment, suggesting that the relaxation induced by HC extract was mainly endothelium-dependent, NO-mediated and only partially endothelium-independent, and NO-independent. To clarify whether NO is responsible for the endothelium-dependent relaxation, further investigation is required as to whether pretreatment with other inhibitors, such as oxyhemoglobin (a NO trapper) and
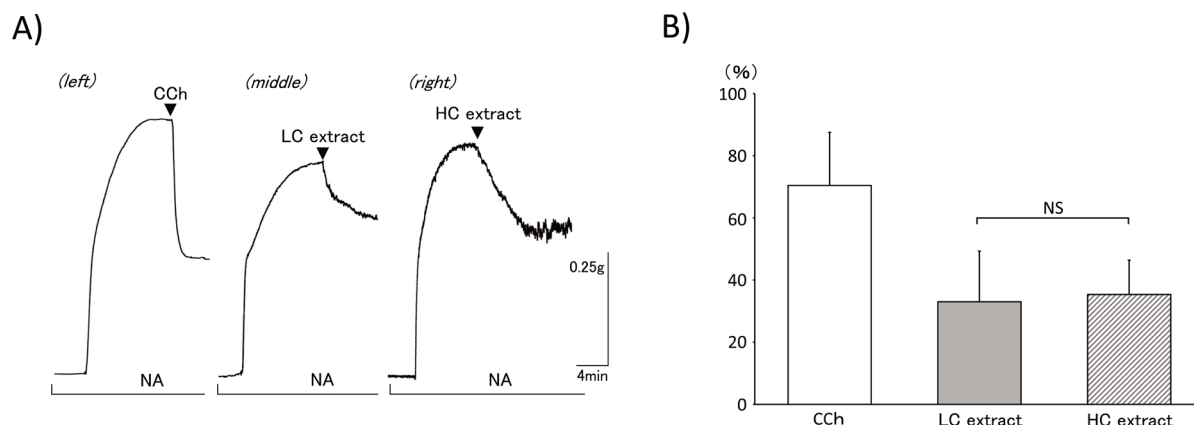
Fig. 1. Heartworm (HW) extract caused relaxation of isolated rat thoracic aortic strips with endothelium. (A) Representative changes in aortic strip tension, produced by HW extract treatment. An aortic strip was mounted in a 5 m*l* organ bath filled with Tyrode's solution. After noradrenaline (NA; 0.1 μM)-induced contraction reached plateau levels, 50 μl HW extract was injected into the bath solution and incubated for 6 to 18 min. Each graph is a representative single observation, with horizontal lines indicating contraction elicited by NA. The force-displacement transducer output is an arithmetic scale. (*Left*) Endothelium integrity was confirmed by generation of carbamylcholine chloride (CCh; 100 μM)-induced relaxation. Specimens that had more than 50% relaxation of the NA-induced contraction were designated as being aortic strips with functional endothelium. (*Middle*) A low concentration of HW extract (LC extract) caused sustained relaxation of the aortic strip. (*Right*) A high concentration of HW extract (HC extract) also caused relaxation of the aortic strip. (B) The size of relaxation is expressed as a percentage of the NA-induced contraction. Bar graphs represent the mean ± SD from 10 (open bar, CCh), 8 (solid bar, LC extract) and 5 (hatched bar, HC extract) strips, respectively. NS indicates not significant.
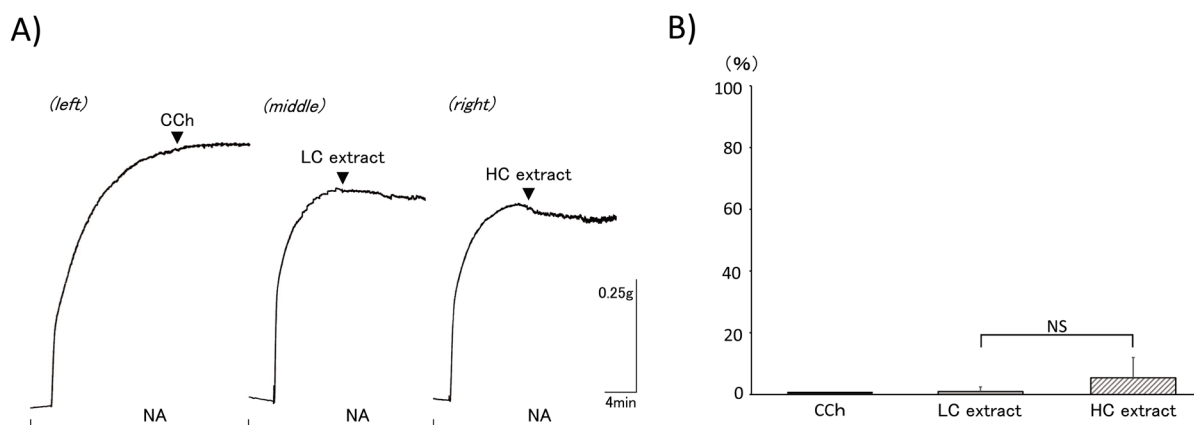

Fig. 2. Removal of the endothelium abolishes or reduces HW extract-induced relaxation in isolated rat thoracic aortic strips. (A) Representative tension changes in endothelium-denuded aortic strips produced by HW extract treatment. Experimental protocols are the same as for Fig. 1. (*Left*) Inability of CCh to relax aortic strips was judged to indicate denudation of the endothelium. (*Middle*) LC extract abolished relaxation. (*Right*) HC extract dramatically reduced relaxation, although there was still slight relaxation in some endothelium-denuded strips. (B) The extent of relaxation is expressed as a percentage of the NA-induced contraction. Bar graphs represent the mean ± SD from 8 (open bar, CCh), 5 (solid bar, LC extract) and 7 (hatched bar, HC extract) strips, respectively. NS indicates not significant.

methylene blue (a guanylate cyclase inhibitor), inhibits relaxation. Furthermore, to verify NO involvement in HW extract-induced relaxation, the Griess assay [18] could assess the accumulation of the NO degradation products, nitrate and nitrite, in the organ bath filled with Tyrode's solution. In order to elucidate the mechanism of HC extract induced, endothelium-independent relaxation, further studies are needed to determine whether the effect might be associated with decreased intracellular calcium brought about by either: a) an inhibition of extracellular calcium influx via Ca^{2+} and K^{+} channels or b) an inhibition of intracellular calcium release through the specific inositol triphosphate and ryanodine receptor channels [12, 13].

Our previous study demonstrated that HW extract had the ability to cause both contraction and relaxation of isolated canine abdominal aortic strips [10]. LC extract consistently elicited vasoconstriction of the canine abdominal aorta, irrespective of the presence of endothelium, whereas HC extract elicited relaxation and contraction of the canine aorta with and without endothelium, respectively [10]. This study showed, however, that both LC and HC extracts could not induce vasoconstriction of the isolated rat
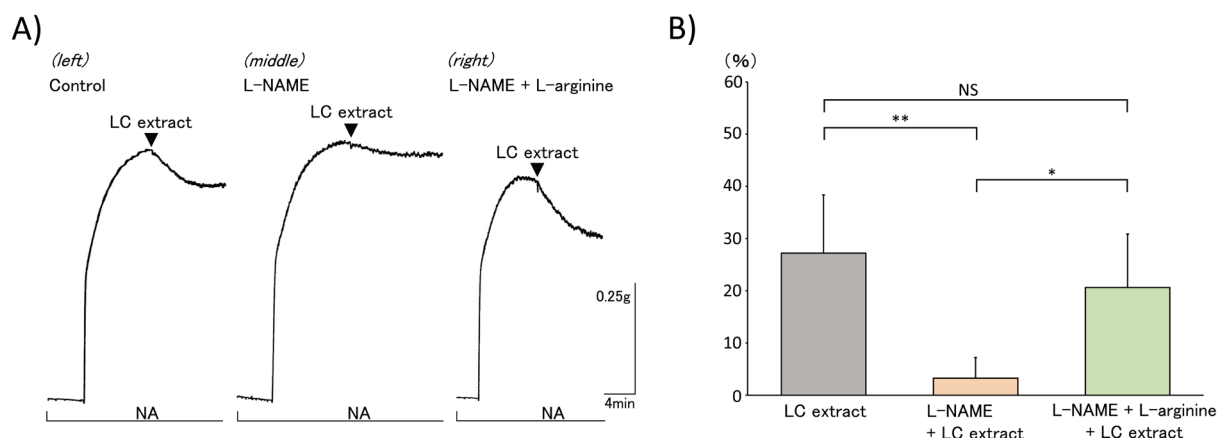
Fig. 3. The effect of N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) on rat aortic strips with endothelium undergoing relaxation induced by LC extract. (A) Representative tension changes in aortic strips produced by LC extract. Experimental protocols are the same as for Fig. 1. (*Left*) LC extract relaxed aortic strips (control). (*Middle*) Treatment with 300 μM L-NAME blocked this relaxation. (*Right*) The blockade by L-NAME was reversed by additional treatment with 30 μM L-arginine. L-NAME and L-arginine were added to the organ bath 30 min before addition of NA. (B) The degree of relaxation is expressed as a percentage of the NA-induced contraction. Bar graphs represent the mean ± SD from 5 (solid bar, LC extract), 5 (orange bar, L-NAME + LC extract) and 3 (green bar, L-NAME + L-arginine + LC extract) strips, respectively. ** $P<0.01$ and * $P<0.05$ calculated by one-way ANOVA followed by the Tukey-Kramer method. NS indicates not significant.
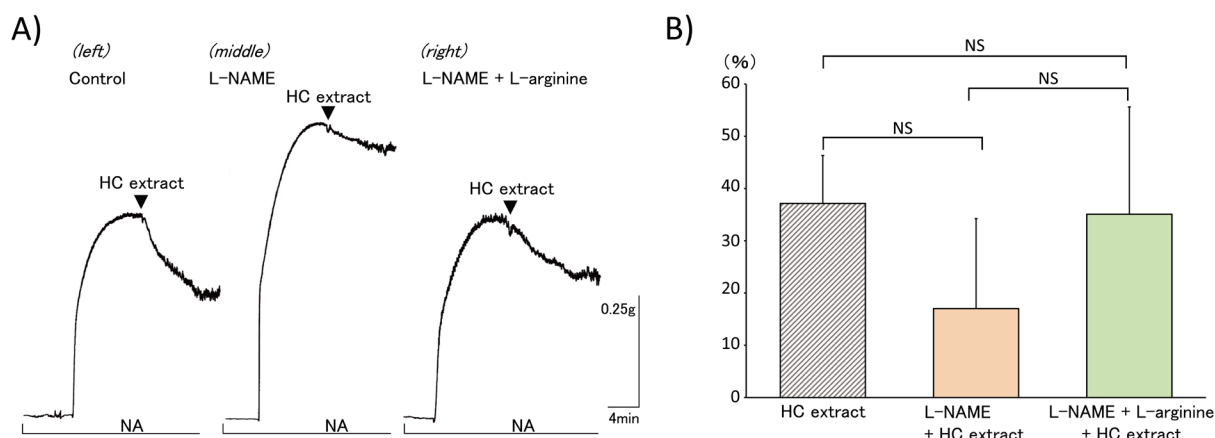

Fig. 4. The effect of L-NAME on rat aortic strips with intact endothelium undergoing relaxation induced by HC extract. (A) Representative tension changes in aortic strips produced by HC extract. Experimental protocols are the same as for Figs. 1 and 3. (*Left*) HC extract relaxed aortic strips (control). (*Middle*) Treatment with 300 μM L-NAME slightly reduced the relaxation. (*Right*) The reduction by L-NAME in these strips was reversed by additional treatment with 30 μM L-arginine. (B) The degree of relaxation is expressed as a percentage of the NA-induced contraction. Bar graphs represent the mean ± SD from 4 (hatched bar, HC extract), 4 (orange bar, L-NAME + HC extract) and 3 (green bar, L-NAME + L-arginine + HC extract) strips, respectively. NS indicates not significant.

thoracic aorta, irrespective of the presence of endothelium. The most likely explanation is that the rat thoracic aorta is less sensitive than the canine abdominal aorta to the induction of vasoconstriction by HW extract. Relaxation induced by the HW extract was observed after NA-induced pre-constriction, and thus, another possible explanation for the results is that the HW extract was unable to induce further constriction of rat thoracic strips.

Our experiments were conducted using a whole body extract from adult HWs to determine, with certainty of the vasoactive effects of HW components on rat thoracic aorta. The effects of metabolic products released from live adult worms in pulmonary arteries remain unexamined. In addition, the whole body extract prepared from adult female HWs comprises the microfilaria components and molecules of *Wolbachia* [16]. The symbiont bacterium *Wolbachia* is present in the HW body and has been implicated in the modulation of host inflammatory and immune responses during infection [21]. Therefore, the results observed in this study might also arise from the effects of microfilaria and *Wolbachia* components on the rat thoracic aorta.

In conclusion, we have demonstrated that whole body extract from adult HWs induces rat thoracic aorta relaxation *in vitro* and that a bioassay using isolated rat thoracic aortas may be a useful tool for purifying and identifying the relaxation-inducing substances in the HWs [10]. More studies are required to determine whether excretory/secretory metabolic products from live adult

HWs, or components from microfilaria or *Wolbachia*, might be responsible for this aortic relaxation. In addition, further studies are needed to elucidate the signaling mechanisms, involved in HW extract-induced relaxation of rat thoracic aorta, and may include endothelial NO synthase phosphorylation [2, 3].

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