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Interaction of endothelial eccrine mechanisms and human adrenomedullin on vascular resistance in canine bone

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Abstract

Adrenomedullin is a novel peptide known to be one of the most potent vascular smooth muscle relaxing agents in vivo. The aim of this study is to investigate the effect of adrenomedullin in relation to nitric oxide, prostaglandins and endothelium-derived hyperpolarized factor (EDHF). A 0.1-ml bolus of 1 nmol human adrenomedullin is a potent inhibitor of the pressor response to exogenous norepinephrine infusion in an ex vivo canine tibia perfusion model for a duration of at least 70 min ($P < 0.005$). This attenuation of vascular smooth muscle contraction occurs even when nitric oxide production is blocked by N^G-monomethyl-L-arginine acetate (L-NMMA) infusion and EDHF is blocked by tetraethylammonium infusion, although the effect is of shorter duration (at least 10 min). Indomethacin as well does not affect the suppression of norepinephrine-induced vascular smooth muscle contraction. Based on these data, human adrenomedullin has both nitric oxide- and EDHF-dependent mechanism as well as a nitric oxide- and EDHF-independent mechanism. © 1998 Elsevier Science B.V.

Keywords: Adrenomedullin; EDHF (endothelium-derived hyperpolarized factor); Nitric oxide (NO); Prostaglandin; Vascular resistance

1. Introduction

A relatively new vascular smooth muscle relaxing agent, adrenomedullin, is a peptide originally discovered in human pheochromocytoma. It is known to be one of the most potent vasodilators in vivo (Kitamura et al., 1993). Adrenomedullin causes a decrease in total peripheral resistance and blood pressure without affecting cardiac output and heart rate in vivo (Hao et al., 1994; Ishiyama et al., 1993). Since cardiac output and heart rate are not altered during the marked systemic vasodepressor response to adrenomedullin, activation of the adrenomedullin vasodilator mechanism may represent a therapeutic opportunity in the clinical management of ischemia/reperfusion injury as well as hypertensive diseases.

The presence of ischemia-associated reperfusion injury following free vascularized bone transplantation or tissue replantation adversely affects the result of these procedures. Resulting ischemic damage to the bone vasculature is a possible explanation for the 15% failure rate and significant incidence of non-union observed in free vascularized fibular grafts (De Boer and Wood, 1989). The incidence of the so-called 'no-reflow phenomenon' is thought to increase after longer periods of global tissue ischemia. Efforts to maximize bone blood flow following a period of transient ischemia may therefore be warranted and may improve the success rate for microvascular bone transfer. We previously have reported that a bolus injection of adrenomedullin results in vascular smooth muscle relaxation in a canine tibia ex vivo perfusion model (Kato et al., 1996a). This observation also occurs in the absence of vascular endothelium (Kato et al., 1996b). It is therefore plausible that adrenomedullin may be a useful therapeutic adjunct to improve tissue blood flow following surgical revascularization, particularly in the presence of ischemia-associated reperfusion injury.

Adrenomedullin possesses 21% homology with the amino acid sequence of human calcitonin gene-related peptide-alpha (human CGRP-alpha), and adrenomedullin responses are attenuated in the presence of 30 nM CGRP[8–37], a CGRP1 receptor antagonist (Entzeroth et al., 1994; Nuki et al., 1993). Adrenomedullin is also known to induce nonadrenergic and noncholinergic vasodilation (Nuki et al., 1993) and increase cAMP levels in vascular smooth muscle cells (Ishizaka et al., 1994). The

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mechanism of action of adrenomedullin is not well clarified. Recently it has been demonstrated that vascular endothelium produces adrenomedullin (Sugo et al., 1994), in addition to other vascular smooth muscle relaxing factors such as nitric oxide, prostacyclin and EDHF. It is of significant clinical and pharmacologic interest to elucidate the mechanism of action of adrenomedullin and to study the relationship between adrenomedullin and other vasodilator substances produced by vascular endothelium. We therefore have investigated the effect of adrenomedullin on norepinephrine-induced pressor responses in the presence of L-NMMA (nitric oxide synthase inhibitor), indomethacin (cyclo-oxygenase inhibitor) and tetraethylammonium (potassium channel blocker) using a canine tibia ex vivo perfusion model.

2. Materials and methods

2.1. Canine tibia isolation

Adult mongrel dogs (weight 23–28 kg) of either sex were anesthetized (pentobarbital, 30 mg/kg of body weight) and killed by exsanguination. Both tibiae were excised. After removal of the fibula, the tibial nutrient artery was identified on the posterolateral surface of the proximal tibial metaphysis. Close to the nutrient foramen, the nutrient artery was separated from the accompanying nutrient vein and nutrient nerve. The nutrient artery was then cannulated with a 0.965-mm external diameter polyethylene cannula (Clay Adams, Parsippany, NJ, USA) with the aid of optical magnification and microsurgical instruments (Davis and Wood, 1992; Davis et al., 1992; Dean et al., 1992; Kato et al., 1996a,b; Ye et al., 1993). All periosteal and muscular branches of the nutrient artery were ligated and both tibiae isolated in an extra-periosteal manner from soft tissue attachments.

2.2. Ex vivo perfusion apparatus

A technique of ex vivo perfusion was performed as described previously (Davis and Wood, 1992; Davis et al., 1992; Dean et al., 1992; Kato et al., 1996a,b; Ye et al., 1993). Each tibia was placed in a humidifier. The nutrient artery cannula was connected to a constant-flow roller pump (Miniplus 2; Gilson, Middleton, WI, USA) and perfused with modified Krebs–Ringer (KR) solution (composition, mM: NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.7; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; CaEDTA, 0.026; glucose, 11.1) at 37°C. KR solution was prepared daily and aerated (95% O_2 and 5% CO_2) to produce a pH of 7.45. The perfusion pressure was measured continuously by a strain-gauge pressure transducer (Spectra-Med DTX/Plus; Gould, Cleveland, OH, USA) and recorded by a pen recorder (Allen Datagraph, Model 2125 E; Salem, NH, USA). An initial flow rate was set at 0.5 ml/min for 40 min. Following this, the flow rate was sequentially elevated to 1.0, 1.5, 2.0 and 2.5 ml/min at 20-min intervals. Using this preparation, we have confirmed by prior studies that a flow rate at 2.5 ml/min results in optimal distension of the vessel wall to produce maximal smooth muscle contractile response (Kato et al., 1996a).

2.3. Drug preparation

Adrenomedullin (Phoenix Pharma, Mountain View, CA, USA), norepinephrine, indomethacin, tetraethylammonium chloride (all Sigma, St. Louis, MO, USA) and N^G-monomethyl-L-arginine acetate (L-NMMA; Calbiochem–Novabiochem, La Jolla, CA, USA) were used in this study. Tetraethylammonium is an inhibitor of Ca^{2+}-activated K^+ channels which blocks the effect of EDHF.

2.4. Experimental protocols

A schematic representation of the experimental design is illustrated in Fig. 1. Twenty-four adult mongrel dogs were utilized and divided equally into four groups. One of each paired tibiae from six dogs was selected at random for Groups 1–4A, and the other for Groups 1–4B.

Group 1A: Adrenomedullin injection + 0.1 ml/min KR solution perfusion

Group 1B: KR solution injection + 0.1 ml/min KR solution perfusion

Group 2A: Adrenomedullin injection + 0.1 ml/min L-NMMA perfusion

Group 2B: KR solution injection + 0.1 ml/min L-NMMA perfusion

Group 3A: Adrenomedullin injection + 0.1 ml/min indomethacin perfusion

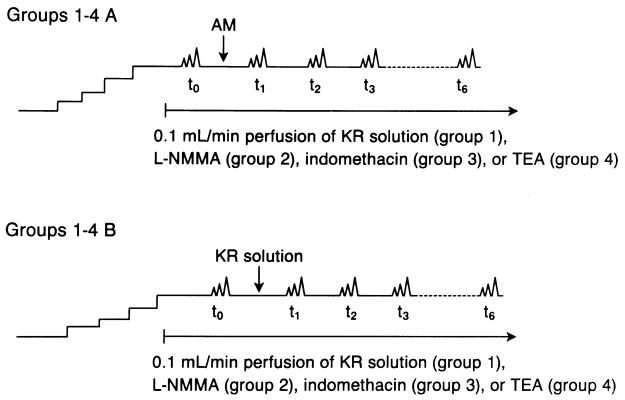

Fig. 1. The time-course of method in the current paper. t_1 NE dose response curve is generated 10 min after AM or KR solution injection. t_1-t_6 have 30 min intervals. KR solution, N^G-monomethyl-L-arginine acetate (L-NMMA), indomethacin or tetraethylammonium (TEA) started to perfuse 10 min prior to t_0 dose response curve and continued to the end of the experiment.

Group 3B: KR solution injection + 0.1 ml/min indomethacin perfusion

Group 4A: Adrenomedullin injection + 0.1 ml/min tetraethylammonium perfusion

Group 4B: KR solution injection + 0.1 ml/min tetraethylammonium perfusion

Following stabilization of the baseline perfusion pressure at a flow rate of 2.5 ml/min to both tibiae, 0.1 ml/min perfusion of KR solution (Group 1), L-NMMA (Group 2), indomethacin (Group 3) or tetraethylammonium (Group 4) was added into the perfusate of both tibiae. The inhibitors were infused to bring the perfusate to a concentration of 10^{-5} M for L-NMMA and indomethacin and 3×10^{-4} M for tetraethylammonium. Previous studies have demonstrated that these doses exhibit predictable inhibitory activity on endothelium-dependent vascular smooth muscle relaxation (Katusic et al., 1987; Palmer et al., 1988; Campbell et al., 1996). Ten minutes following this step, a standard norepinephrine dose response curve was generated (64, 128, and 256 pmol) using 0.1-ml bolus injections into the perfusate (t_0). The summation of the maximal perfusion pressure rise for the three doses was arbitrarily defined as the maximum vascular smooth muscle contraction (100% contraction). Following this, a 0.1-ml bolus injection of 1 nmol adrenomedullin (Groups 1–4A) or a bolus injection of 0.1 ml KR solution (Groups 1–4B) was given. Repetitive norepinephrine responses using the same three norepinephrine doses as at t_0 were then generated at 10, 40, 70, 100, 130 and 160 min (t_1–t_6) following adrenomedullin or KR solution injection to determine the time-dependent effects of adrenomedullin on norepinephrine-induced pressor responses.

2.5. Analysis of data

Results of the quantitative studies are expressed as mean values ± S.E.M. The maximum pressure response of each tibia to the three norepinephrine bolus doses was summed. The initial (t_0) norepinephrine dose response was interpreted as a maximal (100%) contraction. Each succeeding set of norepinephrine dose response curves (t_1–t_6) were summed and expressed as a percentage of the t_0 value.

In this model, the baseline perfusion pressure and pressor response to norepinephrine were stable for a period of 70 min in all groups. Beyond 70 min, however, some tibiae demonstrated erratic baseline perfusion pressures and pressor responses to nonrepinephrine in the presence of tetraethylammonium (Group 4). Therefore, only t_1–t_3 was statistically analyzed in this study.

The statistical analysis was carried out using a three-factor (group, treatment, time) ANOVA model with repeated measures on two factors (treatment, time). There were four levels of group: (1) KR, () L-NMMA, (3) indomethacin, (4) tetraethylammonium; and three levels of time: (1) 10 min, (2) 40 min, (3) 70 min. Due to a significant group by treatment interaction and a significant time by treatment interaction, a subsequent analysis was conducted by performing paired *t*-tests on the treatments at each timepoint within each group. In order to guard against the increased error rate associated with this type of multiple testing, the results were adjusted using the Bonferroni correction. *P* values less than 0.05 were interpreted as statistically significant.

3. Results

The mean perfusion pressures of the tibiae in Groups 1–4 are given in Table 1. There were no significant differences between groups.

In Group 1B, the magnitude of the norepinephrine response curve diminished from the initial t_0 value for a period of 160 min (Fig. 2A). These data are consistent with observations on repetitive norepinephrine responses using this model in prior studies (Kato et al., 1996a). This may reflect desensitization to norepinephrine or depletion of substrates for various vascular smooth muscle active agents synthesized in vascular endothelium. The ANOVA *F*-tests failed to show any significant differences due to the main effects of group and time. However, the pressure as a percentage of the pretreatment value following KR solution injection was significantly higher than that following adrenomedullin injection ($P < 0.001$). However, more interestingly, there was a significant group by treatment interaction ($P < 0.001$). It is due to these interactions that separate paired *t*-tests were performed on the treatments at each timepoint within each group. The results from these analyses are presented in Table 2. The amount of suppression of the norepinephrine response curve produced by a 0.1-ml bolus of 1 nmol adrenomedullin was statistically significantly different from that of time-matched controls (Group 1B) during the first 70 min (Fig. 2A) ($P < 0.005$). The residual pressor responses to norepinephrine varied from 35.6 ± 2.9 to 48.8 ± 5.5% at three sampling points between 10 and 70 min. In Groups 2 and 4, a 0.1-ml bolus of 1 nmol adrenomedullin in the presence of L-NMMA or tetraethylammonium attenuated the pressor responses to

Table 1
Results of the perfusion pressure at 2.5 ml/min flow rate (mean ± S.E.M.)

Group	Perfusion pressure (mmHg)
1A	66.2 ± 3.8
1B	64.5 ± 5.3
2A	61.3 ± 5.3
2B	62.2 ± 5.6
3A	64.3 ± 5.7
3B	67.8 ± 6.4
4A	68.4 ± 3.3
4B	68.7 ± 3.5

There is no significant difference between all groups.

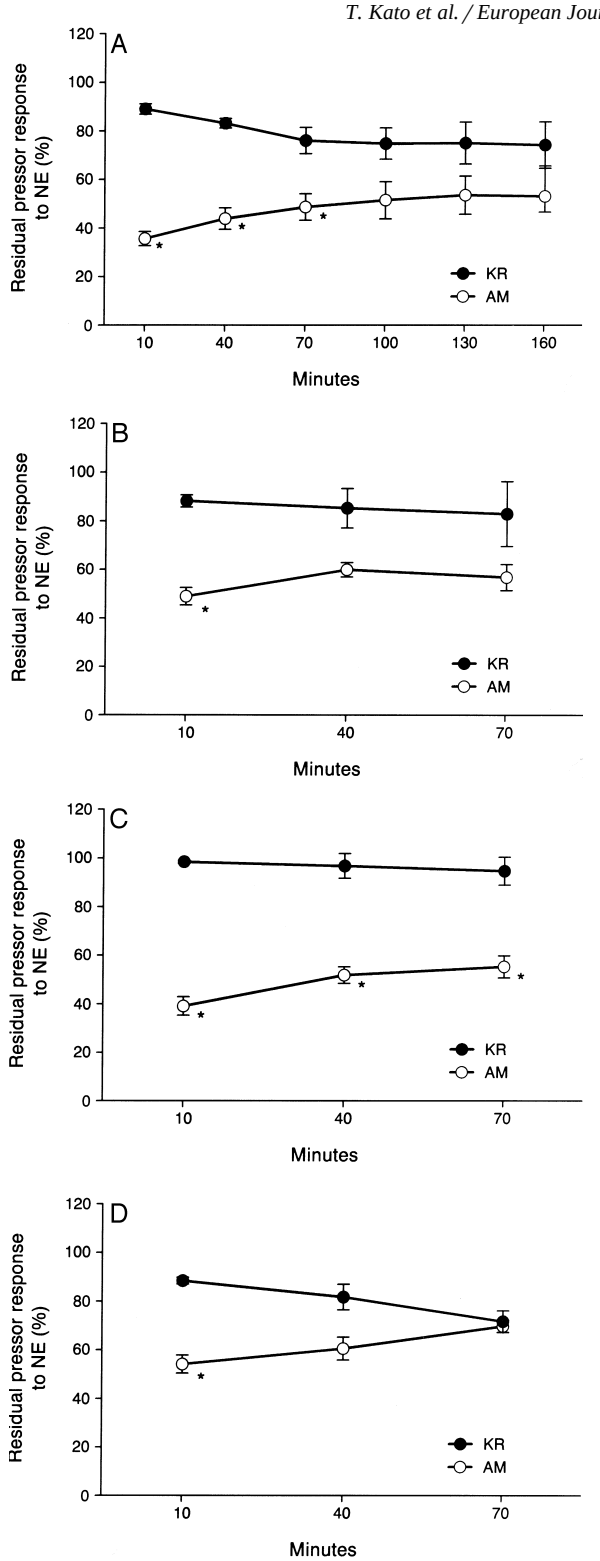

Fig. 2. The effect of a 0.1-ml bolus of 1 nmol AM on the residual pressor response to NE is demonstrated. Flow rate is 2.5 ml/min. 0.1 ml/min perfusion of KR solution (A), L-NMMA (B), indomethacin (C) or TEA (D) is added into the perfusate of both tibiae. ∗Significant difference ($P < 0.05$) of Group 1–4A in relation to Group 1–4B, respectively.

norepinephrine for 10 min (Fig. 2B and D) ($P < 0.05$). The residual attenuation of pressor responses to norepinephrine by adrenomedullin were 49.0 ± 3.6% in Group 2A and 54.2 ± 3.7% in Group 4A at 10 min following a 0.1-ml bolus of 1 nmol adrenomedullin, respectively. Their respective KR controls retained 88.3 ± 3.6% and 88.5 ± 1.4% attenuation, respectively. In Group 3, the magnitude of the reactions to norepinephrine in the presence of indomethacin following a 0.1 ml bolus of 1 nmol adrenomedullin demonstrated no change from the observations of Group 1 (Fig. 2C).

4. Discussion

The present study was designed to investigate the effect of adrenomedullin on the pressor response to exogenous norepinephrine, and to evaluate the mechanism of action of adrenomedullin in relation to nitric oxide, prostaglandins and EDHF productions.

Since Driessens and Vanhoutte described the isolated canine tibia as a useful model for study of the effect of neural, humeral, and metabolic factors on bone circulation, it has been used by many investigators (Driessens and Vanhoutte, 1979; and Davis and Wood, 1992; Davis et al., 1992; Dean et al., 1992; Kato et al., 1996a,b; Ye et al., 1993) and was employed in this investigation. Ex vivo perfusion of the tibia provides excellent control of variables which cannot be controlled in vivo, such as perfusate composition, flow rates, pH, and temperature. However, the limitations of this model must also be recognized and controlled to the extent possible. The vascular smooth muscle contractile responses to serial injections of norepinephrine declines in a predictable manner over time (Fig. 2A). This observation may be explained by a progressive desensitization to norepinephrine or gradual depletion of various substrates required for vascular smooth muscle contraction which is an inherent shortcoming of the ex vivo bone perfusion model. This fact underscores the need for a time-dependent control group using this model. The flow rate in this current study was set at 2.5 ml/min because the optimal flow rate was consistently 2.5 ml/min in our former experiments using an identical bone preparation (Kato et al., 1996a).

In the current study, a 0.1-ml bolus of 1 nmol adrenomedullin attenuated the pressor response to norepinephrine for a duration of at least 70 min. This same observation was made in the presence of indomethacin which suggests that the mechanism of action of adrenomedullin is independent of prostaglandin-mediated pathways. The attenuating effect of adrenomedullin on norepinephrine-induced pressor responses was abbreviated by perfusion of L-NMMA or tetraethylammonium for a 10-min period. In this interval, both L-NMMA and tetraethylammonium perfusion decreased the attenuation effect of adrenomedullin on norepinephrine-induced pressor responses to 49.0 ± 3.6% (Group 2A) and 54.2 ± 3.7% (Group 4A), (control: Group 2B; 88.3 ± 2.5%, Group 4B: 88.5 ± 1.4%). It has previously been reported that

adrenomedullin increases cAMP levels in cultured vascular smooth muscle cells (Ishizaka et al., 1994). Moreover, adrenomedullin demonstrates a vascular smooth muscle relaxing effect following the removal of vascular endothelium (Kato et al., 1996b). It is therefore plausible that adrenomedullin has a direct effect on vascular smooth muscle which results in relaxation. The data herein reported suggest that there are at least three vascular smooth muscle relaxation mechanisms of adrenomedullin all of which are time-dependent. Two of these are nitric oxide- and EDHF-dependent which are effective for a duration of at least 70 min following a 0.1-ml bolus of 1 nmol adrenomedullin. The third mechanism, which has a duration of at least 10 min following a 0.1-ml bolus of 1 nmol adrenomedullin, may be the direct action of adrenomedullin on vascular smooth muscle. These results demonstrate that adrenomedullin is effective in improving flow in the absence of endothelium-mediated nitric oxide and EDHF mediated mechanisms of action. Adrenomedullin may therefore have potential clinical use in instances of ischemia-associated/reperfusion injury where vascular endothelial cell function is impaired. Many of the known vasoactive substances require the presence of intact vascular endothelium to exhibit their effect. Examples include acetylcholine (De Mey et al., 1982), adenosine triphosphate, adenosine diphosphate (De Mey and Vanhoutte, 1981) calcium ionophore A23187 (Furchgott, 1983), substance P (Furchgott and Vanhoutte, 1989), bradykinin (Chand and Altura, 1981) and histamine (Van de Voorde and Leusen, 1983). This endothelium dependence may not be the case with adrenomedullin. Moreover, if the reperfused tissue maintains some intact vascular endothelium, administration of adrenomedullin may both modulate local vascular tone by its direct smooth muscle effect as well as relax vascular smooth muscle by inducing nitric oxide and EDHF release.

In conclusion, the results of the present study suggest that human adrenomedullin has a potent and long-lasting effect which attenuates the pressor response to exogenous norepinephrine in the perfused canine tibia vascular bed. The present data also suggest that human adrenomedullin has multiple time-dependent mechanisms of action. Two of these are nitric oxide- and EDHF-dependent mechanisms. The third is a direct action on vascular smooth muscle which has a more abbreviated action than the endothelium-dependent mechanisms. There is no evidence to suggest that the mechanism of action of adrenomedullin is related to prostaglandin mediated pathways.

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