Original Research

Sympatholytic delta-2 opioid receptors moderate ganglionic vasomotor control

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Abstract

This study tested the hypothesis that enkephalin increases femoral vascular conductance via the delta-2 phenotype of the opioid receptor (DOR-2) within peripheral sympathetic ganglia. Graded pulses of methionine–enkephalin (ME) were administered (0.03–10 μg/kg) into the terminal aorta of anesthetized dogs proximal to lumbar arteries that perfuse vasomotor ganglia regulating femoral blood flow. Femoral vascular conductance increased sharply (ED50 = 2.6×10^{-9} mol/kg) accompanied by declines in arterial pressure and femoral vascular resistance. A dose-related increase in arterial pressure preceded each subsequent fall in pressure. The DOR-2 antagonist, naltriben (NTB), abrogated the hyperemic effect of ME (ID50 = 1.4×10^{-9} mol/kg). DOR-1 blockade (BNTX) was five-fold less effective. The hyperemic effect of ME was also enhanced when sympathetic activity was reflexly increased by bilateral carotid occlusion. The DOR-2 agonist, deltorphin II, produced exaggerated increases in conductance compared with ME that were also reduced by DOR-2 blockade. DOR-1 blockade eliminated the initial pressor responses, exaggerated the subsequent depressor response, increased baseline femoral conductance 10-fold and shifted the ME-mediated hyperemic threshold one dose lower from 0.3 to 0.1 μg/kg, providing indirect support for a competing DOR-1-mediated constriction. Extended exposure to DOR-1 blockade lowered the maximal ME increase in conductance by 30%, suggesting that BNTX reduces the available pool of DOR receptors. In summary, enkephalin mediates a robust hyperemic effect through sympatholytic ganglionic DOR-2 receptors and DOR-1 antagonist studies provide indirect evidence for constituent opposition from a proposed DOR-1-mediated sympathotonic constrictor pathway.

Keywords: DOR phenotypes, enkephalins, sympathetic ganglia, vascular conductance

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Introduction

Opioids may contribute to the peripheral vascular collapse associated with circulatory shock since receptor blockade with naloxone, improved arterial pressure and reversed shock-like conditions in a number of experimental models of circulatory shock.[1–7] A series of elegant studies in dogs demonstrated that leucine-enkephalin produced hypertensive responses in conscious dogs and hypotensive responses in the same animals following anesthesia.[8] Thus, the opioids produced a pressor response in conscious animals when resting sympathetic tone is low and a depressor response in anesthetized animals when sympathetic tone is higher. A peripherally restricted opioid antagonist blocked these effects indicating the target opioid receptor was within reach of the peripheral circulation. The systemic administration of a variety of opioid peptides including enkephalins and dynorphins produced similar sharp declines in blood pressure in anesthetized dogs.[9] Much like the effect of anesthesia, when carotid reflexes were employed to abruptly increase sympathetic activity, the magnitude of the enkephalin-mediated hypotensive response was likewise increased suggesting that enkephalins lowered blood pressure by opposing sympathetic activity. Further support for the sympatholytic connection was provided by the observation that enkephalin-mediated declines arterial pressure increased progressively during the progressive stress of extended surgical procedures.[9]

Wightman *et al.*[10] demonstrated that enkephalin produced a biphasic change in skeletal muscle blood flow in conscious rabbits. They suggested a direct effect on the vasculature or an indirect effect through the elimination of reflex sympathetic tone. The lumbar sympathetic chain ganglion receives blood through lumbar arteries branching off the terminal aorta. Gibbins and Morris[11] established an opioid presence

within sympathetic ganglia that appears to increase as one proceeds caudally along the paravertebral chain. Injection of enkephalin into the abdominal aorta just proximal to these segmental arterial branches produced a sharp decrease in skeletal muscle vascular resistance. If the artery supplying the ganglia was occluded during the enkephalin injection, the subsequent increase in muscle blood flow was not observed. Similarly, when enkephalin was injected directly into the femoral vasculature, the hyperemic response was also not observed.[12] In both the dog and rabbit, ganglionic blockade eliminated the enkephalin-mediated increase in blood flow.[10,12] The response was also blocked by the non-selective opioid antagonist, naloxone. Functional opioid receptors within the lumbar sympathetic ganglia thus appear to moderate cholinergic transmission within the ganglion and the subsequent sympathetically mediated muscle blood flow.

Enkephalins modify cholinergic control of heart rate (HR) from targets within the sinoatrial (SA) node where they produce a similar biphasic effect.[13–18] Introduction of enkephalins by microdialysis into the SA node increased cholinergic transmission at femtomolar dose rates and inhibited cholinergic transmission at nanomolar dose rates. The local receptors that mediate the opposing vagotonic and vagolytic phenotypes are pharmacologically assigned the designations delta-1 phenotype of the opioid receptor (DOR-1) and delta-2 phenotype of the opioid receptor (DOR-2), respectively.[13,14,19]

The nature of the lumbar ganglionic opioid receptor is currently undefined but the influence of enkephalins on ganglionic transmission and the biphasic character of reported responses suggested that phenotypic subtypes of the DOR might also modify cholinergic transmission within the sympathetic ganglion.[8–10] Although DOR subtypes are often invoked to explain observed pharmacological differences[20] and excitatory opioid responses are common,[21] the consequent physiology is relatively unexplored.

Obstructive vascular disease and disabling leg pain in aging, diabetic and smoking populations produce progressive immobility, declining perfusion and muscle weakness. Sympathetic chain ganglia transmit vasomotor signals that regulate normal muscle blood flow. Enkephalins lower arterial blood pressure and increase regional blood flow through opioid receptors that moderate cholinergic transmission within these ganglia. Enkephalins in the SA node increase HR by interrupting vagal transmission through activation of the DOR-2 receptor. The following studies evaluated the role of the DOR in femoral vascular control and tested the primary hypothesis that DOR-2 receptors increase femoral vascular conductance by moderating ganglionic transmission and sympathetic vasomotor tone. The studies also collected indirect support for the secondary hypothesis that ultrasensitive ganglionic DOR-1 receptors normally facilitate sympathetic ganglionic transmission and thus sustain peripheral vascular tone.

Methods

The Institutional Animal Care and Use Committee approved all protocols in compliance with the NIH guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 85-23, revised 1996).

Surgical preparation

Mongrel dogs of either gender weighing 15–30 kg were assigned at random to the experimental protocols. The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and initially ventilated mechanically at 225 mL/min/kg with room air. A combination infusion catheter/pressure transducer (Millar Mikro-Tip, Millar Instruments, Houston, TX, USA) was introduced into the right femoral artery and advanced 3–4 inches to a position just upstream of the L5–L6 region. The catheter provided an arterial infusion port just proximal to origin of the L5 segmental arteries as verified by the immediate biological response to peptide infusion. Infusion of enkephalin distal to this ganglionic access point enters the femoral arteries directly but has little effect on femoral vascular conductance.[12] The transducer provided a continuous measure of arterial pressure and HR (PowerLab; ADI Instruments, Colorado Springs, CO, USA). A fluid-filled catheter was inserted into the right femoral vein and advanced into the inferior vena cava to monitor central venous pressure. An electromagnetic flow probe (10 mm) placed around the isolated left femoral artery provided a continuous measure of femoral blood flow with the aid of a model 501 flow meter (Carolina Medical Electronics, East Bend, NC, USA). Figure 1 illustrates the anatomical relationships. The femoral

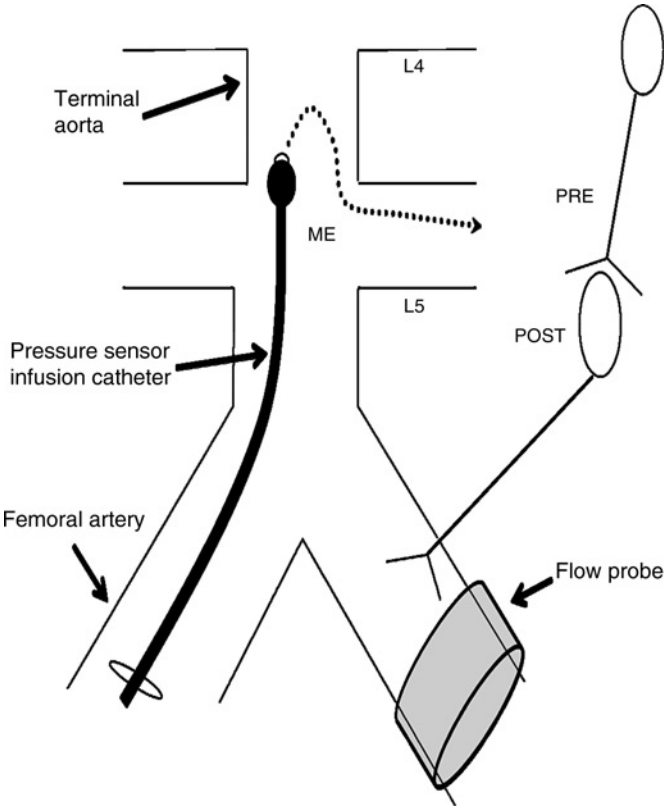

Figure 1 Illustrates the relative position of the infusion catheter, the last lumbar artery, the lumbar sympathetic ganglion and the femoral arterial flow probe

arterial-venous pressure difference and femoral blood flow provided a continuous online recording of both femoral vascular resistance and conductance. Changes in vascular conductance were quantified by integration of the area under the curve for the duration of each deviation from baseline. Surface electrodes recorded a continuous electrocardiogram. The surgeons evaluated the anesthesia regularly and administered supplemental anesthetic as required. The acid–base balance and blood gases were determined with an Instrumentation Laboratories blood gas analyzer (Lexington, MA, USA). The pO_2 (90–120 mmHg), the pH (7.35–7.45) and the pCO_2 (30–40 mmHg) were adjusted to normal by administering supplemental oxygen or bicarbonate or by modifying the minute volume. The carotid arteries were isolated through a ventral midline incision and fitted with adjustable plastic snares to evaluate opioid interactions during sympathetic activation via bilateral carotid occlusion (BCO).

Materials

American Peptide (Sunnyvale, CA, USA) supplied methionine–enkephalin (ME). Tocris Bioscience (Ellisville, MO, USA) supplied TAN-67 (2-methyl 4aa-(3-hydroxyphenyl)-1,2,3,4, 4a,5,12,12aα-octahydroquinolino[2,3,3-g]isoquinoline), BNTX (7-benzylidenaltrexone), naltriben (NTB) and deltorphin II.

Statistical methods

All data were summarized as mean ± standard error of the mean. Within-group comparisons were made with a one-way repeated-measures analysis of variance (ANOVA) or paired *t*-statistic. When the ANOVA indicated differences, multiple comparisons were made *post hoc*, with Tukey's least significant difference test or Dunnett's test. The Student's *t*-test analysis was used to compare selected between-group values. Differences were considered statistically significant when the probability they occurred by chance was $P < 0.05$.

Protocol 1: ME dose response

An initial ME dose response was conducted in each of 12 mongrel dogs. ME was specifically selected relative to other potential DOR agonists because of its very rapid hydrolysis within the vascular space.[9,22] All peptides were introduced into the terminal aorta as bolus injections immediately upstream from the final (L5) pair of lumbar arteries. Once again, injection below this point has no effect on femoral hemodynamics.[12] A standard 5 mL saline flush immediately followed each 1 mL vehicle or peptide injection. An eight-step ME dose response (0.01–30 μg/kg) was constructed. The hemodynamic effect of each dose was recorded and allowed to recover for 5–10 min before exposure to the next higher dose. A BCO was performed and after stabilization of the reflex pressor response at 45 s, a submaximal dose (3 μg/kg) of ME was administered. The occlusion was released (at 90–120 s) once the resulting change in femoral conductance had returned to the preinjection value. On completing the first dose response, the system was permitted to re-equilibrate for 30 min. An identical second dose response was then conducted in a subset of four dogs to access reproducibility and/or the development of tolerance.

Protocol 2: DOR-1 and DOR-2 selectivity

Ten animals were assigned to this protocol. In each case, a submaximal dose of ME (3.0 μg/kg) was pulsed into the terminal aorta at approximately 10-min intervals while recording the femoral conductance. A cumulative dose response to either the DOR-1 antagonist, BNTX or the DOR-2 antagonist, NTB was then constructed in five animals each by administering increasing doses of antagonist into the aorta 5 min prior to each subsequent ME challenge. Antagonist-mediated changes in the basal conductance were recorded prior to each ME challenge. The relative ability of each antagonist to prevent the hemodynamic effects of ME was then determined. The sequential ME challenges were repeated in three animals without the addition of antagonist to verify the temporal consistency of the ME response.

Protocol 3: evidence for DOR-1 participation in ME dose response

Two sequential eight-step ME dose responses (0.01–30 μg/kg) were conducted in eight animals as described in protocol 1. A dose of BNTX (0.3 μg/kg) selective for the DOR-1 receptor was administered 5 min prior to beginning the second ME dose response. This protocol attempted to gather added support for competing DOR-1 activity by demonstrating a shift in the second DOR-2-mediated dose response curve to the left after DOR-1 blockade. A BNTX-mediated leftward shift in the curve would indicate the removal of competition from a difficult to discern decrease in vascular conductance mediated by low doses of ME. The dose of BNTX was based on the earlier dose response and prior work indicating that the canine DOR-1 is far more sensitive to BNTX than the DOR-2 is to NTB.[13,14]

Protocol 4: selective DOR-1 and DOR-2 agonists

After making control measurements in four animals, the native agonist ME (3.0 μg/kg), the selective DOR-1 agonist, TAN-67 (3.0 μg/kg) and the selective DOR-2 agonist, deltorphin II (0.3 μg/kg) were tested in sequence at 10–20-min intervals. The doses of each were selected from prior experience and preliminary experiments. Then the DOR-2 character of the deltorphin response was tested by administering the selective DOR-2 antagonist, NTB (10.0 μg/kg) and then re-administering the same dose of deltorphin.

Results

Baseline cardiovascular indices

Table 1 summarizes the resting blood pressure and HR values for each protocol. No differences were observed when comparing initial values among the four protocols. There were also no significant changes in the starting blood pressure or HR during the course of each individual

Table 1 Resting cardiovascular indices

Protocol 1: ME–ME dose response (n = 4)									
Dose (μg/kg)	**Saline/saline**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	110 ± 7	107 ± 4	107 ± 5	108 ± 4	108 ± 4	110 ± 3	109 ± 5	109 ± 4	110 ± 4
	102 ± 1	101 ± 1	100 ± 2	102 + 2	103 + 2	103 ± 1	104 + 1	105 ± 1	106 + 2
HR (bpm)	113 ± 6	110 ± 7	110 ± 7	109 ± 6	107 ± 5	108 ± 5	109 ± 5	109 ± 6	109 ± 6
	96 ± 4	95 ± 4	95 + 4.5	95 ± 5	94 ± 4.5	94 ± 4	93 ± 3	93 ± 3	93 ± 4
Protocol 2a: NTB (DOR-2 antagonist) dose response (n = 5)									
Dose (μg/kg)	**Saline**	**ME 3.0**	**NTB 0.3**	**ME 3.0**	**NTB 1.0**	**ME 3.0**	**NTB 10.0**	**ME 3.0**	
MAP (mmHg)	97 ± 3	97 ± 4	98 ± 3	99 ± 3	99 ± 4	98 ± 4	104 ± 3	102 ± 4	
HR (bpm)	118 ± 10	121 ± 12	123 ± 11	123 ± 15	121 ± 14	118 ± 11	124 ± 12	120 ± 13	
Protocol 2b: BNTX (DOR-1 antagonist) dose response (n = 5)									
Dose (μg/kg)	**Saline**	**ME 3.0**	**BNTX 0.3**	**ME 3.0**	**BNTX 1.0**	**ME 3.0**	**BNTX 10.0**	**ME 3.0**	
MAP (mmHg)	96 ± 2.5	94 ± 2	97.5 ± 2	98 ± 3	98 ± 2	98 ± 2.5	98.5 ± 3	99 ± 3	
HR (bpm)	133 ± 6	132 ± 7	130 ± 6	130 ± 6	129 ± 6	129 ± 7	129 ± 6	127 ± 7	
Protocol 3: ME–ME dose response (n = 4)									
Dose (μg/kg)	**Saline/saline**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	110 ± 7	110 + 7	107 + 5	108 + 4	108 + 4	108 + 5	109 + 5	109 + 6	109 + 6
	102 ± 1	101 + 1	100 + 2	101 + 3	103 + 2	103 + 1	104 + 2	105 + 1	106 + 1
HR (bpm)	113 ± 6	110 ± 7	110 ± 7	109 ± 6	108 ± 5	108 ± 5	109 ± 5	109 ± 6	109 ± 6
	96 ± 4	95 ± 4	95 ± 5	95 ± 5	94 ± 5	95 ± 4	93 ± 3	93 ± 3	93 ± 3
Protocol 4: ME–ME + BNTX (n = 5)									
Dose (μg/kg)	**Saline/BNTX 0.3**	**ME 0.01**	**ME 0.03**	**ME 0.1**	**ME 0.3**	**ME 1.0**	**ME 3.0**	**ME 10.0**	**ME 30.0**
MAP (mmHg)	102 ± 3	101 ± 2	102 ± 1	103 ± 1	104 ± 1	104 ± 1	105 ± 1	106 ± 2	106 ± 3
	103 ± 2	105 ± 4	105 ± 3	105 ± 4	106 ± 3	105 ± 4	106 ± 3	105 ± 4	105 ± 2
HR (bpm)	112 ± 7	111 ± 6	111 ± 6	111 ± 5	110 ± 5	109 ± 5	109 ± 6	112 ± 7	107 ± 7
	100 ± 6	99 ± 6	99 ± 6	98 ± 7	98 ± 6	97 ± 7	96 ± 6	97 ± 7	91 ± 2
Protocol 5: Sal-ME-TAN-67-Delt II-NTB-Delt II (n = 4)									
Dose (μg/kg)	**Saline**	**ME 3.0**	**TAN-67 3.0**	**Delt II 0.3**	**NTB 3.0**	**Delt II 0.3**			
MAP (mmHg)	107 ± 5	107 ± 6	109 ± 6	110 ± 6	104 ± 5	103 ± 5			
HR (bpm)	117 ± 16	115 ± 15	114 ± 14	116 ± 14	113 ± 13	114 ± 13			

ME, methionine-enkephalin; MAP, mean arterial pressure; HR, heart rate; NTB, naltriben; DOR-2, delta-2 opioid receptor; DOR-1, delta-1 opioid receptor

protocol. The second line of values in protocols 1, 3 and 4 are for the second of the two dose responses.

Study 1: ME–ME repeated dose response

The injection of ME gradually increased femoral vascular conductance as the dose increased with an apparent threshold between 0.3 and 1.0 μg/kg, an ED50 of 2.0 μg/kg and a maximum near 10 μg/kg (Figure 2). The last five doses were different from the vehicle. Temporal changes in the contributory factors HR, mean arterial pressure (MAP), femoral arterial blood flow and femoral arterial resistance were evaluated. HR (not shown) was unchanged at any dose. Although significant arterial pressure, femoral conductance and femoral resistance responses were observed at multiple doses, only the 10 μg/kg dose is illustrated in each case for the sake of clarity. An injection artifact produced a consistent 3–5 mmHg increase in arterial pressure at 10 s that returned to baseline at 20 s. Arterial pressure following ME injection followed a consistent biphasic pattern. The initial rise during the first 30 s rose above and was sustained beyond the injection artifact and then quickly declined to a nadir 30–40 s later in the response (Figure 3a). Although not illustrated, both phases of the response increased progressively as the dose of ME increased and both the hypertensive and hypotensive effects were significant at the highest doses. This suggests that as each dose arriving at the target rises, a small low-dose, sympathotonic effect of ME precedes the more prominent sympatholytic effect that evolves when the full dose reaches the target. The dose responses for increasing femoral blood flow and declining femoral vascular resistance (Figures 3b and c) followed similar dose relationships to that observed for conductance. The changes in flow and resistance were more sensitive to the agonist and were significant at all ME doses tested, supporting the assertion that the change in resistance was primary event. Flow increased rapidly to its highest value between 40 and 50 s. Dose-dependent declines in arterial pressure consistently trailed the changes in peripheral vascular hemodynamics

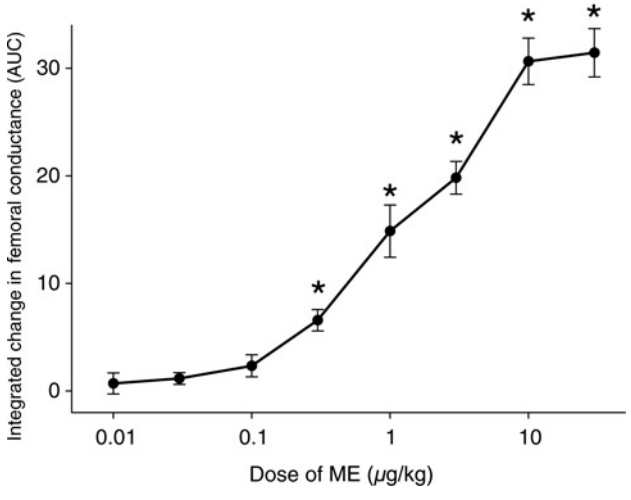

Figure 2 Illustrates integrated changes in femoral conductance mediated by increasing bolus injections of ME (0.01–30.0 μg/kg) into the terminal aorta. The symbol (*) indicates the change in the conductance was significantly different from control at $P < 0.05$. A repeated-measures ANOVA was used for the analysis and values are means and standard error of the mean for 12 subjects. ME, methionine-enkephalin; ANOVA, analysis of variance

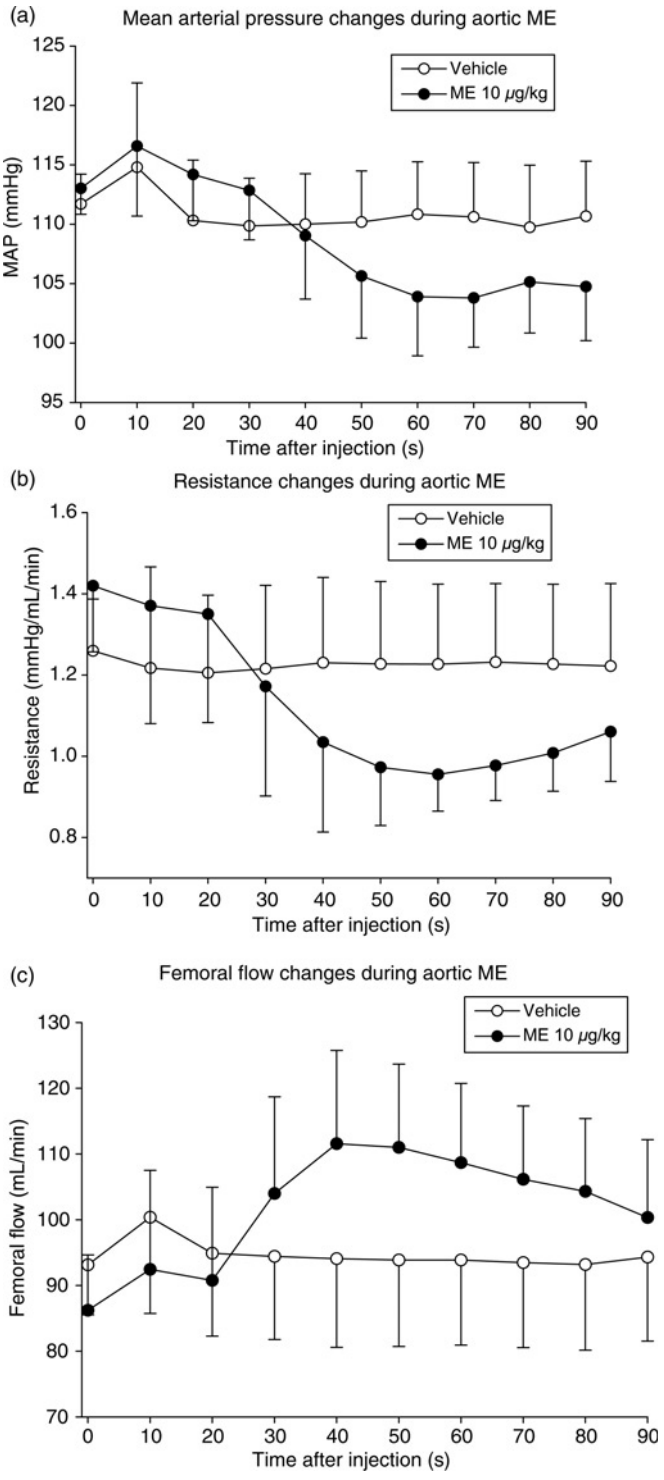

Figure 3 Illustrates changes in mean arterial pressure, femoral resistance and femoral flow, respectively for 90 s after the injection of either vehicle or ME (10 μg/kg) into the terminal aorta. Tracings for the lower doses are omitted for clarity. The statistical analysis employed a repeated-measures ANOVA and changes in arterial pressure were significant for the four highest doses and all doses were significant for resistance and flow. Values are means and standard error of the mean for 12 subjects. ME, methionine-enkephalin; ANOVA, analysis of variance

by 10–20 s, reinforcing the primacy of the effect on arterial resistance. The subsequent declines in arterial perfusion pressure presumably moderated the magnitude and duration of the change in flow. After 30 min washout and equilibration, the dose response was repeated in four animals to evaluate for tachyphylaxis. The two dose responses were comparable and not statistically different (not illustrated). The very short half-life of the peptide and the widely spaced dosing intervals may have minimized the rapid down regulation of opioid responses commonly observed with longer lived alkaloid opioids like morphine. The carotid arteries were occluded bilaterally (BCO) just below the carotid sinuses to increase sympathetic activity. This produces a reversible increase in peak HR (125 ± 5 versus 136 ± 4 bpm $P < 0.001$), mean arterial blood pressure (102 ± 3 versus 124 ± 5 mmHg, $P < 0.001$), femoral blood flow (61 ± 7 versus 81 ± 10 mL/min, $P < 0.001$) with surprisingly little change in femoral vascular resistance (1.93 versus 1.86, NS). When ME was administered during the BCO, the increase in femoral conductance was increased by approximately one-third compared with the pre-BCO response, reinforcing the sympathetic dependence and sympatholytic character of the hyperemic response (Figure 4).

Study 2: DOR-1 and DOR-2 selectivity

Study 2 was designed to test the hypothesis that ganglionic DOR-2 receptors are responsible for the enkephalin-mediated increases in conductance. This was accomplished by comparing repeated ME-mediated changes in conductance in the presence of increasing DOR-1 or DOR-2 blockade, respectively, with BNTX or NTB. A submaximal dose of ME (3.0 μg/kg) selected from the prior dose response curve was repeatedly administered into the terminal aorta at 10–15-min intervals. In each case, the response returned to baseline prior to the administration of the next dose. In three animals, the ME was administered five times at 10-min intervals without any other intervention as a repeated-injection/time control. The effect of ME was unchanged through five sequential injections (not illustrated). In the remaining animals, increasing doses of antagonist were administered into the aorta and equilibrated for 5 min prior to each subsequent ME challenge. The effects of DOR blockade on the integrated changes in conductance during each sequential ME injection are illustrated in Figure 5a. The DOR-2 antagonist, NTB (filled circles) quickly attenuated the increase in conductance with an approximate threshold of 0.3 μg/kg and an ID50 of 0.7 μg/kg (1.4×10^{-9} mol/kg). The threshold and ID50, respectively, for the DOR-1 antagonist, BNTX (open circles) were three and six times higher at 1.0 and 4.5 μg/kg (8.6×10^{-9} mol/kg). Combining ME with the lowest dose

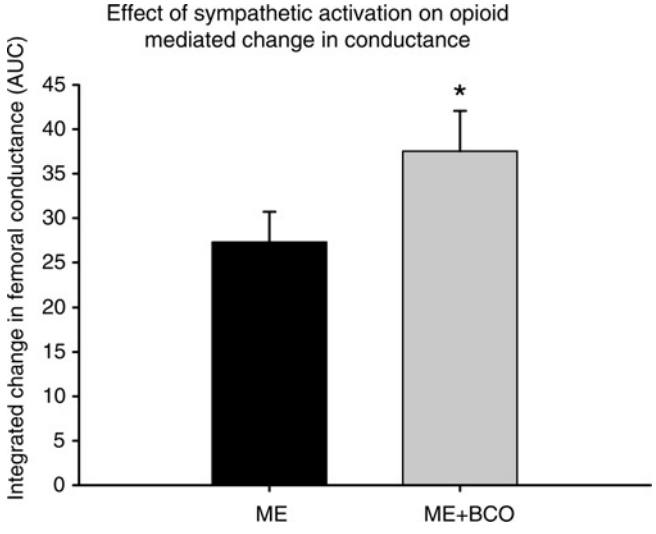

Figure 4 Compares ME (3.0 μg/kg)-mediated increases in femoral vascular conductance during resting hemodynamic conditions and during reflex sympathetic activation mediated by 90–120 s of a BCO. Baseline conductance prior to ME administration was lower during the BCO. Values are means and standard error of the mean for 12 subjects; (*) indicates a significant difference from ME during resting conditions, $P < 0.05$. A repeated-measures ANOVA was employed for the comparison. ME, methionine-enkephalin; BCO, bilateral carotid occlusion; ANOVA, analysis of variance

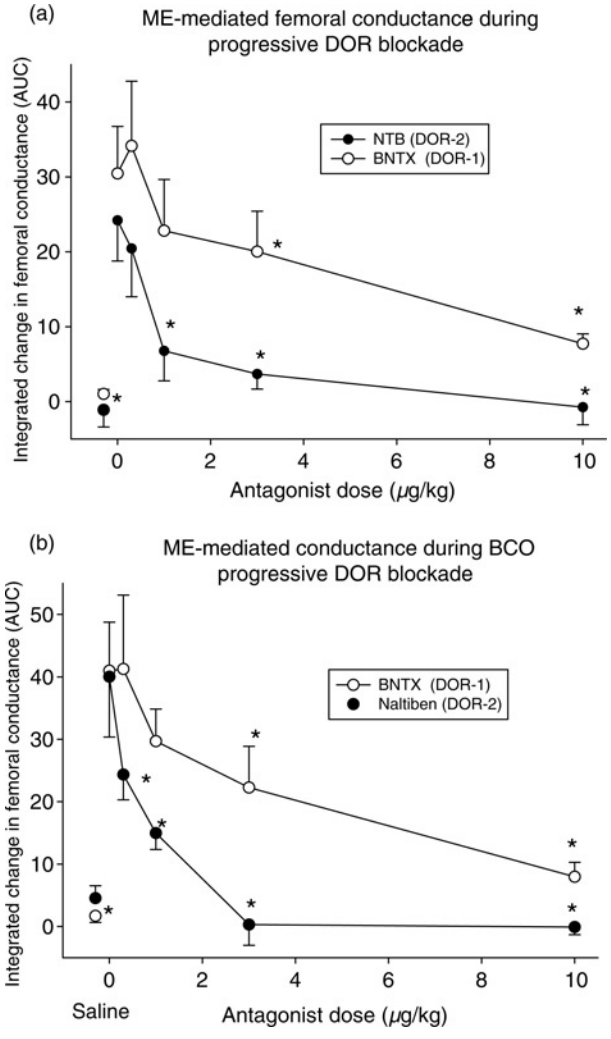

Figure 5 Illustrates the effects of the DOR-1 and DOR-2 blockade on ME-mediated increases in femoral vascular conductance, respectively, under resting hemodynamic conditions (a) and during reflex sympathetic activation (b). Values are means and standard error of the mean for five subjects in each group. A repeated-measures ANOVA was used to determine differences within each of the two experiments and the symbol (*) indicates significant differences from ME alone. DOR-1, delta-1 opioid receptor; DOR-2, delta-2 opioid receptor; ME, methionine-enkephalin; ANOVA, analysis of variance

of BNTX produced a consistent, small, but non-significant increase in conductance during ME injection in agreement with the proposed removal of a DOR-1-mediated constrictor activity. BNTX has a much higher affinity for the canine DOR-1 receptor than its counterpart NTB has for the DOR-2 receptor. Based on prior studies, BNTX should saturate all DOR-1 receptors at the lowest dose employed.[13,14] This suggests that the decline in conductance at higher doses of BNTX is not the result of DOR-1 blockade since the receptor should have been saturated at much lower doses. Other data that follow suggest that the BNTX-mediated reduction in conduction at higher doses is not competitive inhibition of either DOR-1 or DOR-2 receptors but a time-dependent sequestration of DOR-1 receptors at the expense of the total DOR pool. NTB alone had no effect on baseline femoral conductance. However, the lowest two doses of BNTX (0.3 and 1.0 μg/kg) produced, respectively, five- and 10-fold transient increases in the baseline conductance (Figure 6) suggesting again that BNTX removed a constitutive DOR-1-mediated constriction at rest. The transient character suggests that compensatory mechanisms quickly restored the resting flow.

The effect of enkephalin and selective DOR blockade were evaluated again during reflex sympathetic activation. A BCO was conducted after each dose of antagonist and ME was retested at the midpoint of the 90–120 s BCO. Enkephalin produced a 30% greater increase in conductance when administered during sympathetic activation. NTB had no effect on the steady-state HR (121 ± 13 versus 115 ± 12 bpm, NS), blood pressure (101 ± 6 versus 106 ± 7 mmHg, NS), femoral blood flow (49 ± 6 versus 50 ± 6 mL/min, NS) and femoral vascular resistance (2.1 ± 0.22 versus 2.2 ± 0.21, NS). The subsequent changes in hemodynamics were also unaltered by NTB (e.g. MAP 128 ± 9 versus 133 ± 10 mmHg and blood flow 69 ± 16 versus 73 ± 10, NS). NTB blocked the ME-mediated change in conductance with an ID50 near 0.7 μg/kg and was thus similar to that observed in the absence of increased sympathetic activation (Figure 5b). DOR-1 blockade similarly had no effect on resting or post-BCO hemodynamics (e.g. MAP pre 105 ± 4 versus 107 ± 4 and post 125 ± 9 versus 131 ± 8 mmHg, NS). The effect of BNTX on integrated conductance was also very similar to that observed under resting sympathetic conditions. The failure of the initial dose of BNTX (0.3 μg/kg) to reproduce even a suggestion of the modest ME-mediated increase in conductance noted in Figure 5a may be the result of the much higher ambient sympathetic tone and already high ME-mediated flow response during the BCO.

Study 3: evidence for DOR-1 participation in ME dose response

This study systematically evaluated the presence of opposing DOR-1-mediated sympathetic facilitation during the DOR-2 conductance dose response curve. By eliminating baseline DOR-1 activity, BNTX should shift the ME conductance curve to the left. The dose of BNTX (0.3 μg/kg) was selected since it did not acutely reduce the ME response in study 2. The initial ME dose response curve (Figure 7) was comparable with the dose responses presented in Figure 2. The four highest doses of ME were all significantly different from the baseline saline injection. After 30 min equilibration, a single dose of BNTX was administered and the ME dose response was repeated. When the dose

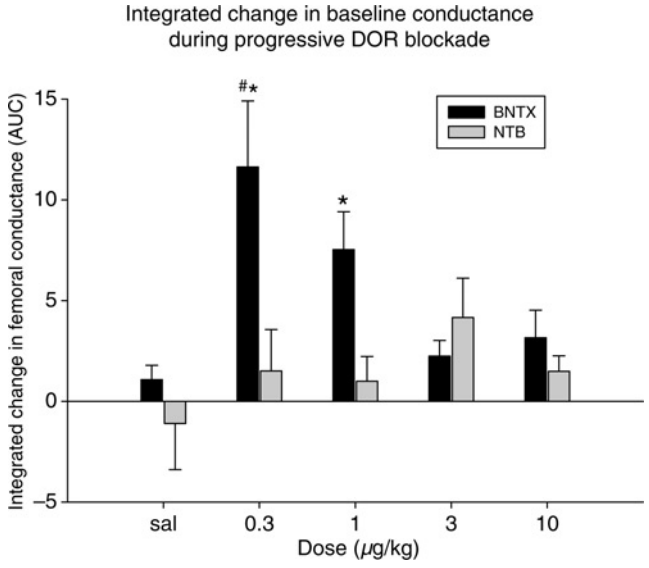

Figure 6 Illustrates the effects of BNTX and NTB alone on the integrated changes in femoral conductance under baseline conditions. Values are means and standard error of the mean. The symbol (#) indicates a significant increase in femoral conductance at 0.3 μg/kg of BNTX compared with all other doses of BNTX. The symbol (*) indicates a significant increase compared with control/saline. A repeated-measures ANOVA was used to determine differences within each of the two experiments and the values are means and standard error of the mean for seven subjects in each group. NTB, naltriben; ANOVA, analysis of variance

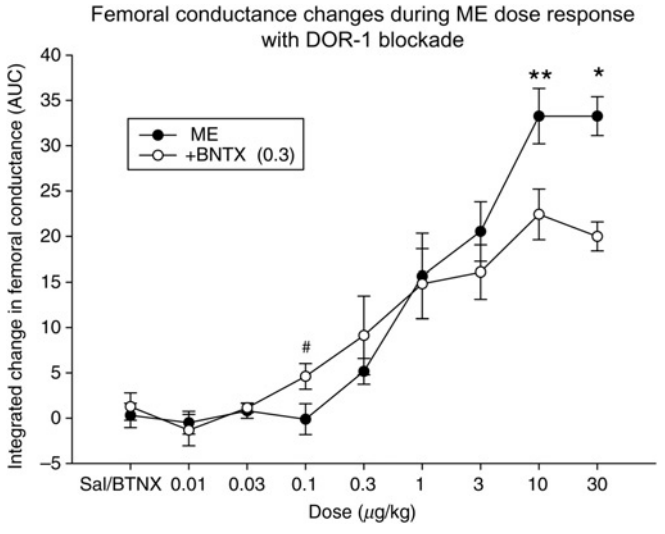

Figure 7 The figure illustrates the effect of DOR-1 blockade with BNTX on changes in femoral conductance during the ME dose response. The symbol (#) indicates the change in the femoral conductance was significantly different from prior dose. The symbol (*) indicates the value was different from the paired dose after BNTX at $P < 0.05$. Individual point comparisons were made with the aid of a paired *t*-statistic. Values are means and standard error of the mean for eight subjects with the exception of the 30 μg dose where the number of subjects was three. DOR-1, delta-1 opioid receptor; ME, methionine-enkephalin

response reached 0.1 μg/kg, a consistent though very modest increase in conductance was apparent compared with the prior dose. Thus, the threshold for increasing conductance in this group of animals was one dose earlier after DOR-1 blockade. The initial leftward shift was, however, not maintained, and the rising curve tapered off early and finished significantly below the initial dose response by approximately 25%. This dose of BNTX did not reduce conductance in study 2 when the ME was tested shortly after BNTX. In this second case, the ganglia were exposed to BNTX for the entire length of the dose response, 80 min or more. When given sufficient exposure to BNTX, the later parts of the ME dose response curve were shifted lower, thus reducing the apparent maximum response. This effect suggests that DOR-1 blockade increases sensitivity early and then reduces efficacy later, perhaps by reducing the number of DOR-2 receptors. Furthermore, the decline in the DOR-2 response after extended exposure to BNTX does not appear to be competitive inhibition since its negative influence is greatest at higher doses of agonist and less effective at lower doses when the competitive antagonist/agonist advantage is greatest. BNTX also eliminated the initial increase in arterial pressure during the first 30 s of the ME response. Figure 8 superimposes the ME response from Figure 3a for direct comparison. This comparison also demonstrates that the hypotensive response to ME is greater after removing the competition from the putative DOR-1 constrictor activity. These data provide additional indirect support to the existence of DOR-1-mediated sympathotonic activity at low agonist concentrations and the likelihood that BNTX reduces available DORs through their sequestration as the occupied DOR-1 phenotype.[23]

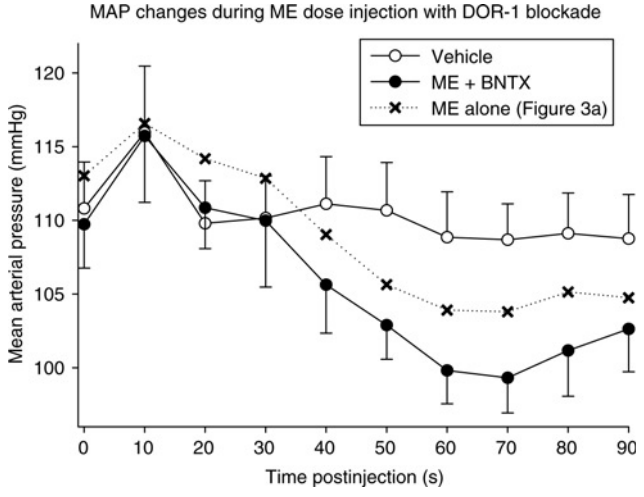

Figure 8 This figure illustrates ME-mediated changes in arterial pressure following DOR-1 blockade with BNTX. Although the higher doses were again significantly different from vehicle ($P < 0.05$), only the 10 μg/kg dose is illustrated for clarity. Note the absence of the early hypertensive response at 20 and 30 s. The dotted line illustrates for comparison the prior mean response at this dose in the absence of DOR-1 blockade (Figure 3a). A repeated-measures ANOVA was used to determine differences and values are means and standard error of the mean for seven subjects. ME, methionine-enkephalin; DOR-1, delta-1 opioid receptor; ANOVA, analysis of variance

Study 4: selective DOR-1 and DOR-2 agonists

Study 4 used subtype selective agents to verify the DOR-2 character of the ME-mediated increase in femoral vascular conductance. The longer half-lives of the selective agents made full dose responses impractical at this time. A submaximal dose of ME (3 μg/kg) produced a consistent reference increase in femoral vascular conductance. The subsequent injection of a near-equimolar dose of the DOR-1 agonist, TAN-67, produced a small increase in conductance that was not different from vehicle and a transient 14 mL/min increase in the peak flow response. The HR (114 ± 15 to 114 ± 15 bpm), arterial pressure (111 ± 6 to 111 ± 6 mmHg) and hindlimb resistance were unchanged. Based on preliminary data, the dose of the DOR-2 agonist, deltorphin II was reduced 10-fold to 0.3 μg/kg. Even at this lower dose, deltorphin increased femoral conductance to nearly double that of ME and with a significantly higher 92 mL/min increase in peak flow than the maximal response observed for ME in study one (25 mL/min). Deltorphin increased HR (108 ± 10 to 114 ± 9 bpm, $P = 0.05$), and decreased arterial pressure (116 ± 5 to 94 ± 5 mmHg, $P = 0.01$) and hindlimb resistance (1.15 ± 0.11 to 0.56 ± 0.09, $P = 0.03$). The response to deltorphin recovered as quickly as that for ME suggesting that the difference in response was not the result slower metabolism or clearance from the target compartment. Following recovery to baseline, an ID90 dose (3 μg/kg versus ME) of the DOR-2 antagonist NTB was administered. NTB had little effect of its own (HR 107 ± 9 to 107 ± 9; MAP 110 ± 4 to 110 ± 5 mmHg) but reduced the integrated response to deltorphin by 60%, reinforcing the DOR-2 character of the sympatholytic effect. While the absolute changes in HR (108 ± 9 to 110 ± 9 bpm, $P = 0.02$), MAP (113 ± 5 to 90 ± 5 mmHg, $P = 0.005$), hindlimb flow (80 mL/min, $P = 0.04$) and resistance (0.98 ± 0.07 to 0.52, $P = 0.005$) were moderated by DOR-2 blockade, they remained significantly different from vehicle (Figure 9). Increasing the dose of NTB threefold (not illustrated) reduced the response to deltorphin by another 5%, reinforcing further the much higher efficacy of deltorphin versus ME and NTB. Thus, the hyperemic effects of the ME were reproduced by the DOR-2 selective agonist, deltorphin, and subsequently reduced by the DOR-2 antagonist, NTB. A 10-fold higher dose of the DOR-1 agonist, TAN-67, was largely ineffective except for a small increase in peak flow that was not different from saline alone.

Discussion

The primary objective of the current study was to evaluate the participation of distinct DOR phenotypes in the ganglionic regulation of muscle blood flow. DOR responses are generally assigned to subtypes based on the ability of the subtype specific pharmaceuticals to replicate or abrogate the response. Index DOR responses were triggered by ME introduced into the descending aorta immediately proximal to the segmental lumbar artery that perfuses the L5-ganglion. This distil ganglion provides the neural regulation of hindlimb blood flow. ME consistently produced

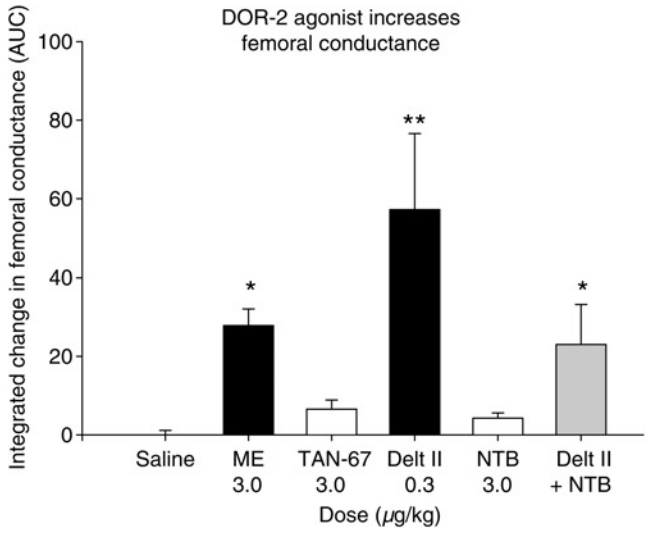

Figure 9 The figure compares the response with ME to the effect of selective DOR-1 (TAN-67) and DOR-2 (Delt II) agonists on femoral conductance. Also illustrated is the ability of DOR-2 (NTB) blockade to reduce the hyperemic effects of the DOR-2 agonist deltorphin II. The symbol (**) indicates the change in the femoral conductance was significantly different from all other treatments. The symbol (*) indicates the change in the femoral conductance was significantly different from the saline. A repeated-measures ANOVA was used to test differences and the values are means and standard error of the mean for four subjects. ME, methionine-enkephalin; DOR-1, delta-1 opioid receptor; DOR-2, delta-2 opioid receptor; NTB, naltriben; ANOVA, analysis of variance

acute changes in arterial pressure and femoral hemodynamics. ME-mediated hyperemic and hypotensive responses were blocked by the DOR-2 antagonist, NTB, and reproduced by the DOR-2 agonist, deltorphin, suggesting that they were mediated by ganglionic DOR-2 receptors. The DOR-1 agents, BNTX and TAN-67, by comparison were far less effective. DOR-1 blockade increased vascular conductance, eliminated an early rise in ME-mediated arterial pressure and enhanced the DOR-2 depressor response. Although less dramatic these later observations suggest potential DOR-1 participation. The gangliolytic DOR-2 involvement is quite clear; however, the proposed participation by opposing DOR-1 receptors remains to be established.

The data presented above indicate that opioid receptors are capable of quickly changing ganglionic transmission and the subsequent distribution of blood flow in the large muscle mass comprising the lower body. The clinical implications for moderating leg blood flow are potentially important. DORs may be important for overriding central constrictor signals and/or reinforcing regional demands for blood flow. Sensory afferent signals from ischemic muscle may orchestrate the regional flow of blood by short circuiting central sympathetic signals at the ganglion. The pharmacological manipulation of this system might be clinically helpful to those with sympathetically mediated hypertension or those with restricted flow or claudication in the lower extremities. The plasticity of opioid receptors suggests that shifts in the mix of ganglionic DOR phenotypes could favor hypertension or orthostatic hypotension. Sympatholytic DOR-2 receptors might explain the contributory role of endogenous opioids in circulatory shock and may be important for determining the character of opioid-based interventions.

Exogenous enkephalins and the associated DORs are capable of quickly modifying sympathetic ganglionic transmission and femoral conductance in dogs and rabbits.[10,12] The present study shows that higher doses of ME produce a robust DOR-2-mediated increase in femoral conduction most likely through the intra-ganglionic inhibition of acetylcholine secretion.[12] This effect would be functionally similar to the vagolytic effect of DOR-2 activation in the SA node.[13,14,17,18] Although the sympathotonic effect of lower dose enkephalin is more difficult to demonstrate, a small progressive increase baseline vascular resistance and subsequent arterial pressure occurred as the dose of ME was increased. DOR-1 blockade eliminated this initial hypertensive response, produced an acute increase in baseline femoral vascular conductance and a small though not significant increase in enkephalin-mediated vascular conductance. Since the hypothesis suggests that the endogenous enkephalin required to stimulate the DOR-1 system is very low, the enkephalin present in anesthetized animals may be sufficient to produce a DOR-1-mediated constriction and mask the expected DOR-1 response to exogenous enkephalin. The pharmacological evaluation of DORs in the canine SA node supports the existence of both DOR-1 and DOR-2 phenotypes in the dog.[13,14] The receptor protein was co-localized with cholinergic nerve terminals and isolated cardiac cholinergic synaptosomes.[24] BNTX and NTB blocked the DOR-1 and DOR-2 responses, respectively. The differences in sensitivities to antagonist were remarkable, with nodal DOR-1 receptors being several orders of magnitude more sensitive to BNTX than DOR-2 receptors were to NTB in the same tissue. The parallels between the nodal and ganglionic findings suggest that similar DOR interactions may be at work at cholinergic junctions within the sympathetic ganglion. In each case, the DOR-2 receptor interrupts cholinergic transmission and is sensitive to blockade by nanomolar doses of NTB. DOR-1 activation in both tissues appears to do the opposite and facilitate cholinergic transmission. Like the vagotonic effect in heart, the sympathotonic effect appears to occur early as the dose of enkephalin in the target rises, is less robust and is sensitive to subnanomolar doses of BNTX. This higher sensitivity of DOR-1 responses may indicate that basal opioid activity within the ganglion normally supports vascular resistance and that higher concentrations increase flow as an acute adjustment to sudden or emergent changes in demand. Increases in basal opioid concentrations and sympathetic tone in the anesthetized animals may contribute to the practical difficulty in demonstrating the sympathotonic response in that model. This concept is supported by observations that enkephalins exert pressor effects in conscious animals when sympathetic activity is low and depressor effects in anesthetized animals when sympathetic activity is high.[8] Thus, repeating these studies in conscious animals may be required to determine the importance of the DOR-1 response.

The third study was designed to demonstrate further the constrictive phenotype of DOR-1 activation by low-dose ME. The studies discussed above clearly demonstrated the

sympatholytic DOR-2 phenotype. However, the hypothetical DOR-1 constrictor response was difficult to demonstrate directly. The ME dose response in the presence of low-dose BNTX sought to demonstrate the constrictor response indirectly by its selective removal that would be evident in greater vasodilation and a shift in the dilator dose response up and to the left particularly at the low end of the ME dose response. BNTX appeared to improve ME-mediated increases in conductance early in the ME dose response and shifted the ME threshold dose, one dose lower (0.1 versus 0.3 μg/kg) as expected. BNTX also eliminated the initial ME-mediated increase in arterial pressure in support of a DOR-1-mediated increase in vasomotor transmission. However, contrary to expectation, the initial leftward shift disappeared two doses later and the curve then shifted downward. The reduction in the apparent maximal response strongly suggests that BNTX reduces the available DOR-2 receptors although a change in postreceptor coupling could also be involved. If simple competitive inhibition of the DOR-2 receptor was at work, BNTX should have been more effective early when the agonist dose was lower and the antagonist/agonist ratio was highest. Furthermore, prior studies have indicated that much higher concentrations of BNTX had little or no influence on DOR-2-mediated vagolytic responses in the canine SA node.[13,14] Thus, BNTX appears to foster the depletion of DOR-2 receptors as the exposure time increases. The same dose of BNTX produced no loss of ME-mediated conduction after 5 min (Figures 5a and b) at the beginning of the antagonist dose response but produced a substantive change after 80 + min (Figure 7). This supports the thesis proposed by Deo *et al.*[23] that the two DOR phenotypes are in dynamic equilibrium and that high-affinity BNTX sequesters the DOR-1 receptors. As DOR-2 receptors gradually shift into the DOR-1 phenotype to re-establish subtype equilibrium, the high-affinity BNTX sequesters replete DOR-1 receptors, slowly depleting the DOR-2 ranks until they are insufficient to produce a maximal DOR-2 response.

The current findings and prior work in the SA node support the hypothesis that DOR receptor trafficking and inter-conversion is physiologically relevant and that plasticity among subtypes may be an important determinant of muscle blood flow.

The fourth protocol used selective DOR agents to verify the DOR-2 character of the hyperemic effect. The DOR-2 agonist, deltorphin was very effective and the DOR-1 agonist, TAN-67, was ineffective. The deltorphin effect was reduced by the DOR-2 antagonist, NTB. The failure of TAN-67 to produce the proposed constrictor response does not rule out a DOR-1-mediated constriction. The high dose of TAN-67 was not selected to demonstrate a sympathotonic influence but to rule out DOR-1 participation in the ganglionolytic effect. The vagotonic effect of enkephalin in the heart wanes with increasing doses.[13] TAN-67 is a racemic mixture and its enantomers are known to moderate one another.[25] Complete dose responses in conscious and anesthetized animals with a variety of DOR-1 agonists such as TAN-67 and DPDPE will be required to verify the existence and importance of the proposed sympathotonic DOR-1 pathway.

The elegant central control of arterial pressure via reflex activation of efferent sympathetic vasomotor pathways is well documented. The endothelial and local neuroendocrine mechanisms that regulate the distribution of blood flow within the target vasculature have also been investigated in significant detail. By comparison, the regulation of blood pressure and/or the distribution of blood flow by moderating ganglionic transmission within the sympathetic ganglion are relatively unexplored. The very existence of the ganglia suggests that these neural intersections are more than mere distribution centers on route to assorted peripheral sympathetic targets. Ganglionic organization is complex and contains terminal fibers positive for a variety of neuromodulators, including enkephalins.[26] Some fibers form characteristic terminal baskets around the cell bodies of postjunctional motor nerves but many others do not. The enkephalin positive nerve endings are widely distributed within the lumbar ganglia and are seldom associated with fibers innervating specific cell bodies. This suggests that their actions may be largely prejunctional. A spectrum of potential ganglionic control mechanisms arises from the fact that the complement of neuromodulators differs among ganglia from different anatomical locations. Enkephalins are more abundant in the lumbar sympathetic chain ganglia[27,28] that are in turn responsible for femoral blood flow. The lumbar concentration of enkephalinergic neurons suggests that ganglionic opioid receptors may be particularly important for regulating the buffering capability of the large reserve of vascular capacitance in the lower limbs.

Significance

Neurovascular complications in diabetic patients commonly involve the lower limbs resulting in a vicious cycle of autonomic neuropathy, painful occlusive claudication and eventual immobility that precipitates inactivity and progressive disability. Neural and vascular diseases evolve slowly and the early events in this progressive decline in function are unclear. Sympathetic vasoconstriction is a major component of blood flow regulation in skeletal muscle. Active vasoconstriction in the lower limbs depends on continued cholinergic transmission of efferent vasomotor signals through the lumbar sympathetic chain ganglia. A number of potential neuromodulators including opioids and their respective receptors[11] populate the vasomotor ganglia responsible for the control of the skeletal muscle blood flow. Opioid receptors can reduce normal ganglionic transmission[12] presumably by lowering acetylcholine release. Although the specific character of these opioid receptors is not that well defined, they may represent an important pharmacological target in populations with poor peripheral vascular circulation. Clinically, the ganglionic DORs may provide an alternate therapeutic target to increase femoral perfusion, reduce leg pain and improve mobility in subject populations with occlusive or spastic peripheral vascular disease. Finally, DOR subtype participation in blood flow in human subjects is largely untested and potential differences in receptors and their distribution will need to be carefully evaluated.

In conclusion, ganglionic DOR-2 stimulation increases femoral vascular conductance and represents a potentially important therapeutic target. Support for an opposing DOR-1-mediated increase in ganglionic transmission although present was indirect, less convincing and thus will require more direct verification. Endogenous enkephalins may mask the DOR-1 effect of exogenously administered enkephalin in anesthetized animals. If true, the more sensitive DOR-1 may be an integral part of the basal ganglionic control of peripheral vascular tone and thus will require a far more extensive characterization. Thus, the importance of this system in conscious animals and those with compromised vascular control due for instance, to age, atherosclerosis or diabetes, remains an important future target.

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REFERENCES

1 Amir S. Opiate antagonists improve survival in anaphylactic shock. *Eur J Pharmacol* 1982;**80**:161–2
2 Carr DB, Bergland R, Hamilton A, Blume J, Kastig N, Arnold M, Martin JB, Rosenblatt M. Endotoxin stimulated opioid peptide secretion; two secretory pools and feedback control in vivo. *Science* 1982;**217**:845–8
3 Curtis MT, Lefer AM. Protective actions of naloxone in hemorrhagic shock. *Am J Physiol* 1980;**239**:LH416–H21
4 Curtis MT, Lefer AM. Beneficial actions of naloxone in splanchnic artery occlusion shock. *Experientia* 1981;**347**:403–4
5 Holaday JW, Faden AI. Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock. *Nat Lond* 1978;**275**:1153–4
6 Shadt JC, York DH. The reversal of hemorrhagic hypotension by naloxone in conscious rabbits. *Can J Pharmacol* 1982;**59**:1208–13
7 Vargish T, Reynolds DG, Gurll NJ, Lechner RB, Holaday JW, Faden AI. Naloxone reversal of hypovolemic shock in dogs. *Circ Shock* 1980;**7**:31–8
8 Sander GE, Kastin AJ, Giles TD. MIF-1 does not act like naloxone in antagonizing the cardiovascular activity of leucine-enkephalin in the conscious dog. *Pharmacol Biochem Behav* 1982;**17**:1301–3
9 Caffrey JL, Wooldridge CB, Gaugl JF. The interaction of endogenous opiates with autonomic circulatory control in the dog. *Circ Shock* 1985;**17**:233–42
10 Wightman JM, Shadt JC, Gaddis. Decreased vascular resistance after inta-arterial injection of Met-enkephalin in the hindquarters of conscious rabbits. *J Pharmacol Exp Ther* 1987;**241**:314–20
11 Gibbins IL, Morris JL. Structure of peripheral synapses: autonomic ganglia. *Cell Tissue Res* 2006;**326**:205–2
12 Caffrey HL, Hong G, Barron B, Gaugl JF. Enkephalin lowers vascular resistance in dog hindlimb via peripheral nonlimb site. *Am J Physiol (Heart Circ Physiol)* 1991;**260**:H286–H392
13 Farias M, Jackson KE, Yoshishige D, Caffrey JL. Bimodal δ-opioid receptors regulate vagal bradycardia in canine sinoatrial node. *Am J Physiol Heart Circ Physiol* 2003a;**285**:H1332–H9
14 Farias M, Jackson KE, Yoshishige D, Caffrey JL. Cardiac enkephalins interrupt vagal bradycardia via δ-2 opioid receptors in the sinoatrial node. *Am J Physiol* 2003b;**284**:H1693–H701
15 Jackson KE, Farias M, Stanfill AS, Caffrey JL. Transient arterial occlusion raises enkephalin in the canine sinoatrial node and improves vagal bradycardia. *Auton Neurosci* 2001a;**94**:84–92
16 Jackson KE, Farias M, Stanfill AS, Caffrey JL. Delta opioid receptors inhibit vagal bradycardia in the sinoatrial node. *J Cardiovasc Pharmacol Ther* 2001b;**6**:385–93
17 Farias M, Jackson KE, Stanfill AS, Caffrey JL. Local opiate receptors in the sinoatrial node moderate vagal bradycardia. *Auton Neurosci* 2001;**87**:9–15
18 Farias M, Jackson KE, Johnson M, Caffrey JL. Cardiac enkephalins attenuate vagal bradycardia: interactions with NOS-1-cGMP systems in canine sinoatrial node. *Am J Physiol Heart Circ Physiol* 2003c;**285**:H2001–12
19 Deo SH, Johnson-Davis S, Barlow MA, Yoshishige D, Caffrey JL. Repeated delta-1 opioid receptor stimulation reduces delta-2 opioid receptor responses in the SA node. *Am J Physiol Heart Circ Physiol* 2006;**291**:H2246–54
20 Zaki PA, Bilsky EJ, Vanderah TW, Lai J, Evans CJ, Porreca F. Opioid receptor types and subtypes: the delta receptor as a model. *Annu Rev Pharmacol Toxicol* 1996;**36**:379–401
21 Sarne Y, Fields A, Keren O, Gafni M. Stimulatory effects of opioids on transmitter release and possible cellular mechanisms: overview and original results. *Neurochem Res* 1996;**21**:1353–61
22 Caffrey JL, Mateo Z, Napier LD, Gaugl JF, Barron BA. Intrinsic cardiac enkephalins inhibit vagal bradycardia in the dog. *Am J Physiol Heart Circ Physiol* 1995;**268**:H848–55
23 Deo SH, Barlow MA, Gonzalez L, Yoshishige D, Caffrey JL. Repeated arterial occlusion, delta-opioid receptor (DOR) plasticity and vagal transmission within the sinoatrial node in the anesthetized dog. *Exp Biol Med* 2009;**234**:84–94
24 Deo SH, Barlow MA, Gonzalez L, Yoshishige D, Caffrey JL. Cholinergic location of delta-opioid receptors in canine atria and SA node. *Am J Physiol Heart Circ Physiol* 2008;**294**:H829–38
25 Tseng LF, Narita M, Mizoguchi H, Kawai K, Mizusuna A, Kamei J, Suzuki T, Nagase H. Delata-1 opioid receptor-mediated antinociceptive properties of a nonpeptidic delta opioid receptor agonist, (-) TAN-67, in the mouse spinal cord. *J Exp Pharmacol Ther* 1997;**280**:600–5
26 Murphy SM, Matthew SE, Rodgers HF, Lituri DT, Gibbins IL. Synaptic organisation of neuropeptide-containing preganglionic boutons in lumbar sympathetic ganglia of guinea pigs. *J Comp Neurol* 1998;**398**:551–67
27 Lindh B, Staines W, Hökfelt T, Terenius L, Salvaterra PM. Immunohistochemical demonstration of choline acetyltransferase-immunoreactive preganglionic nerve fibers in guinea pig autonomic ganglia. *Proc Natl Acad Sci USA* 1986;**83**:5316–20
28 Shimosegawa T, Koizumi M, Toyota T, Goto Y, Kobayashi S, Yanaihara C, Yanaihara N. Methionine-enkephalin-Arg6-Gly7-Leu8-immunoreactive nerve fibers and cell bodies in lumbar paravertebral ganglia and the celiac-superior mesenteric ganglion complex of the rat: an immunohistochemical study. *Neurosci Lett* 1985;**57**:169–74

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