Repeated Arterial Occlusion, Delta-Opioid Receptor (DOR) Plasticity and Vagal Transmission Within the Sinoatrial Node of the Anesthetized Dog

SHEKHAR H. DEO, MATTHEW A. BARLOW, LETICIA GONZALEZ, DARICE YOSHISHIGE, AND JAMES L. CAFFREY[1]

University of North Texas Health Science Center, Department of Integrative Physiology, Cardiovascular Research Institute, Fort Worth, Texas 76107

Brief interruptions in coronary blood flow precondition the heart, engage delta-opioid receptor (DOR) mechanisms and reduce the damage that typically accompanies subsequent longer coronary occlusions. Repeated short occlusions of the sinoatrial (SA) node artery progressively raised nodal methionine-enkephalin-arginine-phenylalanine (MEAP) and improved vagal transmission during subsequent long occlusions in anesthetized dogs. The DOR type-1 (DOR-1) antagonist, BNTX reversed the vagotonic effect. Higher doses of enkephalin interrupted vagal transmission through a DOR-2 mechanism. The current study tested whether the preconditioning (PC) protocol, the later occlusion or a combination of both was required for the vagotonic effect. The study also tested whether evolving vagotonic effects included withdrawal of competing DOR-2 vagolytic influences. Vagal transmission progressively improved during successive SA nodal artery occlusions. The vagotonic effect was absent in sham animals and after DOR-1 blockade. After completing the PC protocol, exogenously applied vagolytic doses of MEAP reduced vagal transmission under both normal and occluded conditions. The magnitude of these DOR-2 vagolytic effects was small compared to controls and repeated MEAP challenges rapidly eroded vagolytic responses further. Prior DOR-1 blockade did not alter the PC mediated, progressive loss of DOR-2 vagolytic responses. In conclusion, DOR-1 vagotonic responses evolved from signals earlier in the PC protocol and erosion of competing DOR-2 vagolytic responses may have contributed to an unmasking of vagotonic responses. The data support the hypothesis that PC and DOR-2 stimulation promote DOR trafficking, and down regulation of the vagolytic DOR-2 phenotype in favor of the vagotonic DOR-1 phenotype. DOR-1 blockade may accelerate the process by sequestering newly emerging receptors. Exp Biol Med 234:84–94, 2009

Key words: DOR phenotypes; opioids; vagal function

Introduction

Although prevention of heart disease is always a primary goal, coronary occlusions are common and the salvage of injured myocardium afterward has been an important research focus for decades. Much of this research effort has centered on ischemic preconditioning protocols (27) and the role of endogenous opioids or their receptors in the process (18, 33–35). Preconditioning refers to the process by which short periods of ischemia reduce tissue damage during later longer periods of ischemia. Although multiple mediators are likely to participate in cardioprotection, reports implicate opioids in virtually every form of cardioprotection including the more recently described variants, remote conditioning and post-conditioning (13, 18, 30). Opioids share second messenger systems with another potential mediator, acetylcholine (39), and furthermore, active parasympathetic control of heart rate significantly improves the prognosis for survival following coronary occlusion in patients (23) and experimental animals (3). Thus, robust parasympathetic systems and selective opioid receptor stimulation might logically converge to improve cardiovascular outcomes following coronary occlusion.

The role of opioids in ischemic preconditioning is largely based on the pharmacological studies in multiple animal models that demonstrated that opioid antagonists

Funding was provided by the Advanced Research Program of the Texas Higher Education Coordinating Board.

[1] To whom correspondence should be addressed at University of North Texas Health Science Center, Department of Integrative Physiology, Cardiovascular Research Institute, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107. E-mail: jcaffrey@hsc.unt.edu

Received August 15, 2008.
Accepted September 24, 2008.

DOI: 10.3181/0808-RM-242
1535-3702/09/2341-0084$15.00

abrogate and added opioids mimic the beneficial effects of ischemic preconditioning (18, 24, 33–35, 46). The actual contributions of endogenous opioids are more complex and much less well understood (21, 28, 40, 41). Changes in endogenous opioids are, however, consistent with the cardioprotective thesis in that coronary occlusion increases available endogenous opioids in the myocardial interstitium (21, 28).

Endogenous opioid peptides function as neuromodulators in a wide variety of biological systems and commonly exert acute influences on function by inhibiting neurotransmitter release. However, those same opioids can also be excitatory (22, 31). This dual capacity was evident during vagal stimulation when enkephalins were introduced directly into the sinoatrial (SA) node by microdialysis. Lower enkephalin infusion rates facilitated vagal transmission and lowered heart rate while higher infusion rates interrupted vagal transmission and raised heart rate (14–17, 20). The delta-opioid receptor type 1 (DOR-1) receptor antagonist BNTX (29) blocked the vagotonic effect and the DOR-1 agonist TAN-67 duplicated it (11). In contrast, the DOR-2 antagonist naltriben blocked the vagolytic effect and the DOR-2 agonist deltorphin reproduced it (15, 17). Thus, the same native enkephalin MEAP was observed to produce dose-dependent results opposing one another in the same target.

Repeated occlusion of the SA node artery increased the recovery of endogenous enkephalin from the SA node (19). Contrary to initial expectations, a clear vagotonic effect emerged after the preconditioning (PC) protocol but only during arterial occlusion when interstitial enkephalin in the SA node was elevated. The facilitory effect of endogenous enkephalin on vagal transmission was consistent with the vagotonic effect of exogenous low dose enkephalin cited above and was likewise blocked by the selective DOR-1 antagonist BNTX (17).

The opposing vagotonic and vagolytic effects of enkephalin are difficult to reconcile since evidence suggests that a single known receptor transcript mediates both responses (1). The functional phenotypes are dynamic and the local membrane environment around the receptor may be important for determining the functional phenotype (2, 8, 9, 21, 38). Adding GM-1, a common constituent of membrane lipid rafts, produced an accelerated decline in DOR-2 responses (9). DOR-1 stimulation produced a similar erosion of the vagolytic DOR-2 response (8) suggesting that the two phenotypes interact. These observations support a working hypothesis that opposing functional phenotypes of the DOR interact and that DOR-1 stimulation increases available GM-1 and shifts the DOR-1/DOR-2 balance in favor of the vagotonic DOR-1 phenotype. Thus, the emergence of DOR-1 stimulation during preconditioning may facilitate parasympathetic transmission by reducing DOR-2 opposition. These dynamics would be consistent with the purported role of the DOR-1 in ischemic preconditioning (33–35).

From a practical standpoint, the nodal artery preconditioning studies described above did not determine whether the vagotonic effect that followed the PC protocol was the result of the conditioning stimulus, the subsequent extended arterial occlusion or a combination of both (21). The status of opposing DOR-2 activity was likewise undetermined. The study that follows tested the hypothesis that preconditioning increases DOR-1 activity at the expense of opposing DOR-2 activity.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee and were in compliance with the NIH guide for the Care and Use of Laboratory Animals.

Surgical Preparation. Nineteen mongrel dogs of either gender weighing 15–25 kg were assigned at random to various experimental protocols. The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated and mechanically ventilated initially at 225 ml/min/kg with room air. Fluid filled catheters were inserted into the right femoral artery and vein and advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer to monitor heart rate and arterial pressure during the remainder of the surgical preparation. The venous line was used to administer supplemental anesthetic. Vital signs and anesthesia were evaluated every 30 mins and supplemental anesthesia (~65 mg/hr) was administered as needed. After completing protocols, the anesthetized animals were euthanized by electrically induced, visually verified, cardiac arrest. The acid-base balance and the blood gases were determined regularly with an Instrumental Laboratories Blood Gas Analyzer (Lexington, MA). 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, bicarbonate or modifying the minute volume.

The right and left cervical vagus nerves were isolated through a ventral midline surgical incision. The nerves were double ligated with umbilical tape to reduce afferent nerve traffic during electrical stimulation. The isolated nerves were then returned to the prevertebral compartment for later retrieval. Surgical anesthesia was carefully monitored, and a single dose of succinylcholine (50 μg/kg) was administered intravenously to temporarily reduce involuntary movements of the thoracic muscles during the 10–15 mins required for electrosurgical incision of the chest. The right heart was exposed through an incision at the third interspace and the costosternal cartilage for ribs 2–5 were severed to permit access to the thoracic cavity. The pericardium was opened and the dorsal pericardial margins were sutured to the body wall to support the heart. The left femoral artery was isolated and a high fidelity catheter pressure transducer (Millar Instruments, Houston, TX) was inserted and advanced into the abdominal aorta to measure heart rate

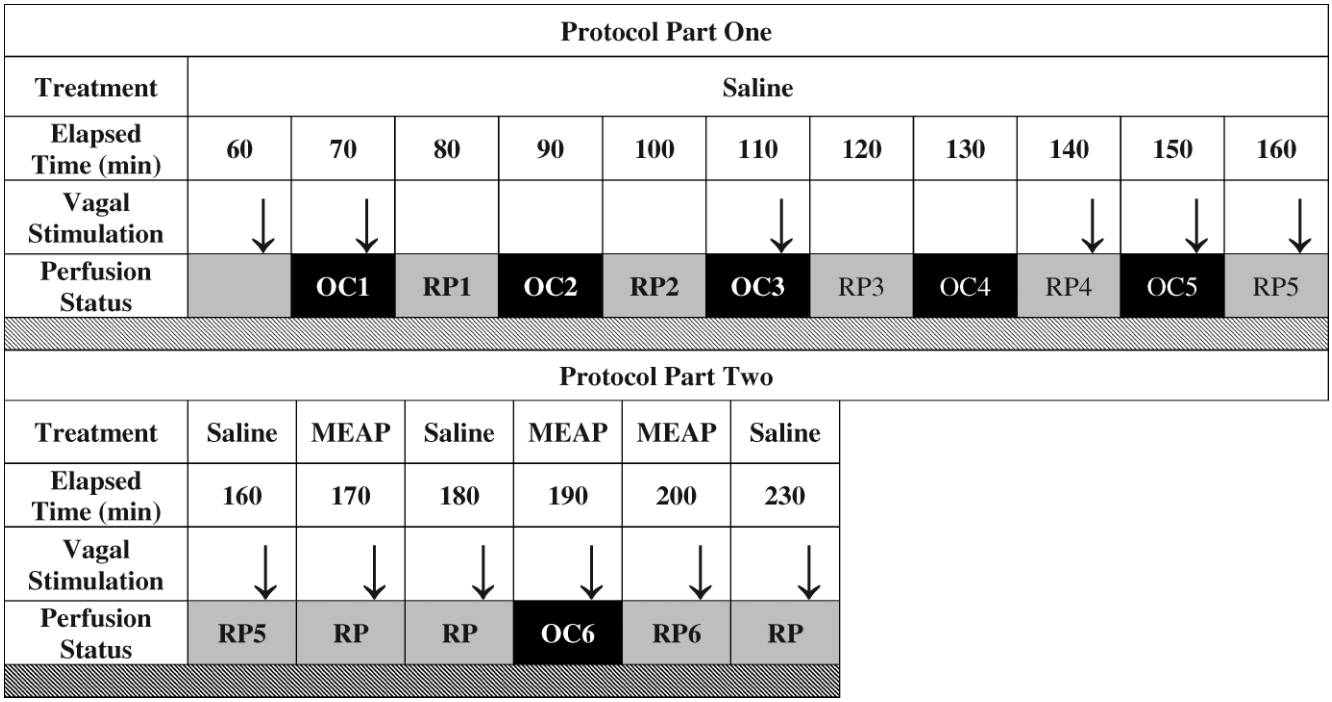

Figure 1. Temporal diagram of parts one and two of the basic experimental protocol. The elapsed time is cumulative and the vertical arrows indicate the times at which the vagus was stimulated. The abbreviations under perfusion status are OC for occlusion and RP for reperfusion.

and blood pressure continuously thereafter online (ADI Instruments, Bella Vista NSW, Australia).

The SA node artery was identified as a branch of the right coronary artery and traced visually to the SA node. A suture was placed loosely around the SA node artery near its origin. The suture was secured with a slipknot to permit the reversible occlusion of the SA node artery as required.

Nodal Microdialysis. The SA node was visualized at the junction of superior vena cava and the right atrium. A 25 gauge stainless steel needle containing a microdialysis line was inserted into the center of the sinoatrial node parallel with the long axis of the node (16, 37). The needle was removed and the probe was then positioned so that the dialysate window was completely immersed within the substance of the sinoatrial node. The microdialysis probe was constructed of a single 1 cm length of dialysis fiber from a Clirans TAF08 (Asahi Medical, Tokyo, Japan) artificial kidney (200 μm ID, 220 μm OD) and hollow 170-μm OD silica glass fiber inflow and outflow lines (SGE, Austin TX). The dialysis tubing permits molecules with a molecular mass of 35,000 kDa or less to cross from the lumen into the nodal interstitium. This technique allows the precise introduction of agents directly into the nodal interstitium for extended periods without provoking complicating systemic reflexes. After placement of the probe in the SA node, the dialysis line was perfused with saline at a rate of 5 μl/min for one hour while the preparation was allowed to equilibrate.

Materials. MEAP (methionine-enkephalin-arginine-phenylalanine) and BNTX (7-benzylidenaltrexone) were obtained respectively from American Peptide (Sunnyvale, CA) and Tocris Bioscience (Ellisville, MO).

Statistical Methods. All data were expressed as mean and standard error of the mean. Differences within subjects were evaluated with repeated measures ANOVA and post hoc analysis was performed with Tukey's test (GB-STAT, Dynamic Microsystems, Silver Springs, MD) for multiple cross comparisons and Dunnett's test was used for multiple comparisons to control. Selected comparisons made between protocols were evaluated with aid of a simple ANOVA followed again by either Tukey's or Dunnett's test as appropriate. In all cases, differences determined to occur by chance with a probability <0.05 were deemed statistically significant.

Protocol 1: Preconditioning Protocol (See Fig. 1). After equilibration, the right cervical vagus nerve was stimulated (Grass S48 stimulator) at a supra-maximal voltage (15 volts) for 15 secs at 3 Hz. This frequency was selected to produce a reproducible sub-maximal decline in heart rate of 30–50 bpm. The 15-secs assessment specifically targets the evaluation of very rapid vagal responses prior to any potential competition from slower sympathetic reflexes. After the basal vagal responses, the SA node artery was temporarily occluded five times for 10 mins each in a PC protocol. The effect of vagal stimulation on heart rate was evaluated by stimulating the right cervical vagus again at the end of the 1st, 3rd, and 5th occlusions just prior to releasing the slipknot as indicated by the vertical arrows in the protocol diagram. After each occlusion the slipknot was released and the artery was perfused normally for 10 mins

before beginning the next occlusion. The stimulation times were selected to minimize the total number of stimulations and to evaluate the effect of occlusion at the beginning, middle and after the end of the PC protocol. The stimulation during the 5th occlusion corresponds in time to the stimulation following the PC protocol at which the vagotonic effect was first observed in earlier studies (15, 19). Control stimulations were conducted during the 4th and 5th reperfusions. After completing the PC protocol (cycle five), the effect of exogenous MEAP on vagal transmission was evaluated three times. MEAP was first added into the dialysis inflow immediately after completing reperfusion five and its vagal test. The dose rate and exposure (1 nmole/min for 5 mins) were based on prior studies (14, 19). The dose was selected from dose response data to produce a submaximal vagolytic effect of near 90% of maximal inhibition and maximize the opportunity to observe a decrement in the vagolytic effect (15, 17, 20). After washing out the MEAP, the restoration of basal vagal transmission was verified. The MEAP infusion was then restarted and a 6th occlusion-reperfusion cycle was conducted during the exogenous MEAP administration to evaluate the vagolytic effect in succession during both occlusion and reperfusion. The vagal-heart rate response was evaluated again before releasing the occlusion and then again after 10 mins of reperfusion. The MEAP was discontinued and washed out for 30 mins. The restoration of basal vagal transmission was verified after the washout interval.

Protocol 2: Sham Preconditioning Protocol (Time Control). The purpose of Study 2 was to determine the potential influence of the duration of the protocol or the repeated vagal stimulation on the subsequent vagal transmission and/or its response to exogenous MEAP. Protocol 2 was identical to protocol 1 except the suture was not tightened at the times corresponding to occlusions 1 through 4 creating a sham PC protocol. Two variants of the protocol were conducted one with and one without an occlusion during stimulation four (OC5). The MEAP infusions and subsequent occlusion/reperfusion six were identical to protocol one.

Protocol 3: DOR-1 Blockade with BNTX Prevents the Preconditioning Effect. The purpose of Study 3 was to determine the potential role of the DOR-1 in the developing vagotonic response and any subsequent changes in the vagolytic, DOR-2 response. Protocol 3 was repeated as described above for protocol 1, except that the specific DOR-1 antagonist BNTX (1 nmole/min) was introduced into the perfusate before beginning the PC protocol and was maintained in the dialysis inflow throughout the remainder of the PC protocol and post-PC protocol. The dose of BNTX employed was supramaximal based on the relative efficacy and selectivity of BNTX versus the vagotonic effect of MEAP in this model system (15).

Results

Baseline Cardiovascular Indices. Table 1 summarizes the resting heart rate and blood pressure throughout each of the three protocols. There was no difference in the initial measure between groups nor was there any change in either variable during the course of any of the three protocols. The apparent trend toward a higher heart rate in the BNTX group was well within historical norms for this model and was not the result of the DOR-1 blockade.

Study 1(a) PC Protocol. Figure 2 illustrates the changes in heart rate observed when the right vagus nerve was stimulated at 3 Hz at the beginning, middle and end of the PC protocol. As demonstrated earlier (21), the series of four short nodal artery occlusions and reperfusions increased the effect of vagal stimulation during the later 5th occlusion. This improvement in nodal vagal transmission clearly required time and/or multiple occlusions to develop since it was not evident after the 1st occlusion but had emerged by the end of the 3rd occlusion. Occlusion alone is thus not sufficient to demonstrate the vagotonic effect. The vagal response observed during the 4th reperfusion though numerically higher was not significantly different from the initial control response. The near restoration of this control response confirms further that the vagotonic response was specifically observed when nodal blood flow is compromised. Contrary to that observation, the stimulation during reperfusion five (after the 5th occlusion) persisted longer suggesting a greater opioid accumulation with successive occlusions (21) and/or a slower washout. This persistent vagotonic effect in RP5 and subsequent washes could of course be due to the progressive accumulation of other metabolites during the sequential occlusions (e.g., adenosine, hydrogen ion) or the establishment of an effect downstream from the opiate receptor that outlives the clearance of the local enkephalins.

Study 1(b) Sham PC Protocol. Figures 3a and 3b illustrate the changes in heart rate in two sham groups in which the vagus was stimulated sequentially without conducting any prior nodal arterial occlusions. The apparent group differences in the initial bradycardia at 3 Hz are not treatment effects but examples of the normal variation in the vagal response observed in this model system. The hatched bars represent stimulations performed at times equivalent to those conducted during occlusions in the prior study. The vagally mediated change in heart rate at each sham occlusion was not different from control stimulations and thus vagal efficacy was unaltered by time or repeated vagal stimulation. In the second group (Fig. 3b), a single occlusion was conducted at a time equivalent to occlusion five to evaluate the combined effect of repeated stimulation, elapsed time and occlusion. The vagal efficiency during that single late occlusion was not different from the preceding or subsequent sham, non-occluded stimulations. Thus, there was no vagotonic effect in the absence of preceding occlusions.

Table 1. Resting Cardiovascular Indices

	Control	OC1	OC3	RP4	OC5	RP5	MEAP	OC6	RP6
Protocol 1: preconditioning protocol ($n = 5$)									
MAP (mmHg)	97 ± 5	101 ± 4	105 ± 5	102 ± 7	104 ± 6	101 ± 7	104 ± 5	94 ± 7	100 ± 8
HR (bpm)	122 ± 5	123 ± 5	126 ± 5	125 ± 5	128 ± 6	127 ± 5	130 ± 6	127 ± 6	131 ± 7
Protocol 2(a): controls: vehicle and duration ($n = 5$)									
	Control	OC1	OC3	RP4	OC5	RP5	MEAP	OC6	RP6
MAP (mmHg)	95 ± 4	95 ± 5	99 ± 4	104 ± 3	102 ± 4	97 ± 4	96 ± 4	99 ± 1	105 ± 6
HR (bpm)	124 ± 7	121 ± 6	120 ± 6	120 ± 7	119 ± 7	118 ± 7	116 ± 6	115 ± 7	116 ± 7
Protocol 2(b): controls: vehicle, duration and OC5 ($n = 4$)									
	Control	OC1	OC3	RP4	OC5	RP5	MEAP	OC6	RP6
MAP (mmHg)	105 ± 2	103 ± 2	104 ± 2	106 ± 2	103 ± 0.4	103 ± 2	107 ± 1	108 ± 2	109 ± 1
HR (bpm)	129 ± 4	124 ± 5	119 ± 5	110 ± 7	110 ± 5	109 ± 7	108 ± 7	106 ± 5	107 ± 6
Protocol 3: DOR-1 mediate preconditioning ($n = 5$)									
	Control	OC1 + BNTX	OC3 + BNTX	RP4 + BNTX	OC5 + BNTX	RP5 + BNTX	MEAP + BNTX	OC6 + BNTX	RP6 + BNTX
MAP (mmHg)	98 ± 7	92 ± 7	95 ± 5	97 ± 3	95 ± 5	94 ± 4	99 ± 3	94 ± 6	98 ± 3
HR (bpm)	144 ± 9	140 ± 9	140 ± 8	138 ± 9	135 ± 9	132 ± 11	134 ± 8	136 ± 8	139 ± 8

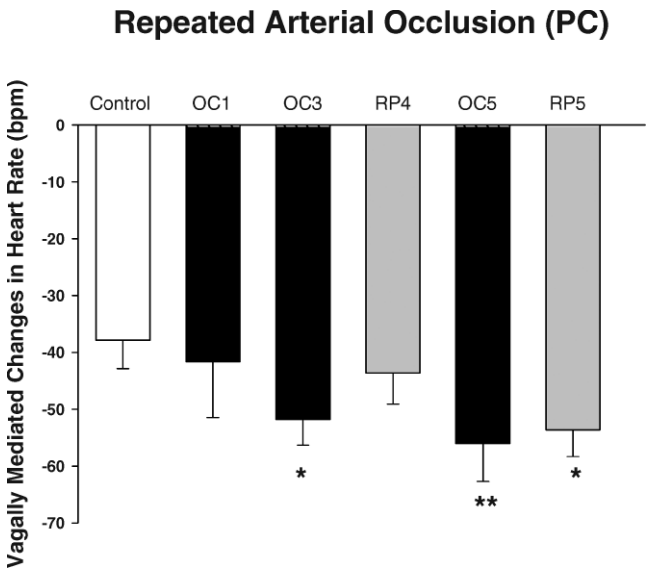

Figure 2. The figure illustrates changes in heart rate mediated by right vagal nerve stimulation at 3 Hz during the sequential occlusion and reperfusion of the PC protocol. Values are means and standard error of the mean for five subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P < 0.05$ and $P < 0.01$, respectively.

Study 1(c) PC Protocol and DOR-1 Blockade. Figure 4 illustrates the changes in heart rate observed when the right vagus nerve was stimulated at 3 Hz at selected times during the PC protocol in the presence of DOR-1 blockade with BNTX. The control response illustrated in the figure was conducted prior to adding BNTX to the dialysate. The results of the subsequent stimulations during the 1st, 3rd, and 5th occlusions were not different from control. Heart rate responses during reperfusions five and six were likewise not different from control. Thus, blockade of the DOR-1 produced a vagal response pattern during the IPC protocol that is indistinguishable from the unoccluded time control (Fig. 3a, b) confirming that the PC mediated vagotonic response was DOR-1 dependent.

Study 2(a) PC and Exogenous MEAP. Study 2 was designed to test whether the vagotonic responses observed during the PC protocol might arise from a reduction in opposing DOR-2 mediated vagolytic responses. As illustrated in Figure 5, a submaximal dose of MEAP was tested to insure a sufficient reserve in which to observe a reduction in the vagolytic response. After completing the five cycle PC protocol, MEAP was tested prior to occlusion. Since the PC protocol produced an increase in vagal efficacy, the degree of inhibition observed was dependent on which reference control was used for comparison. MEAP produced a calculated 21% reduction in the vagal response compared to the original control from the start of the experiment. If the post-washout control was used, the degree of inhibition was closer to 34% for the post-PC control. The MEAP was washed out and restoration of the post-PC-control response was demonstrated. When the

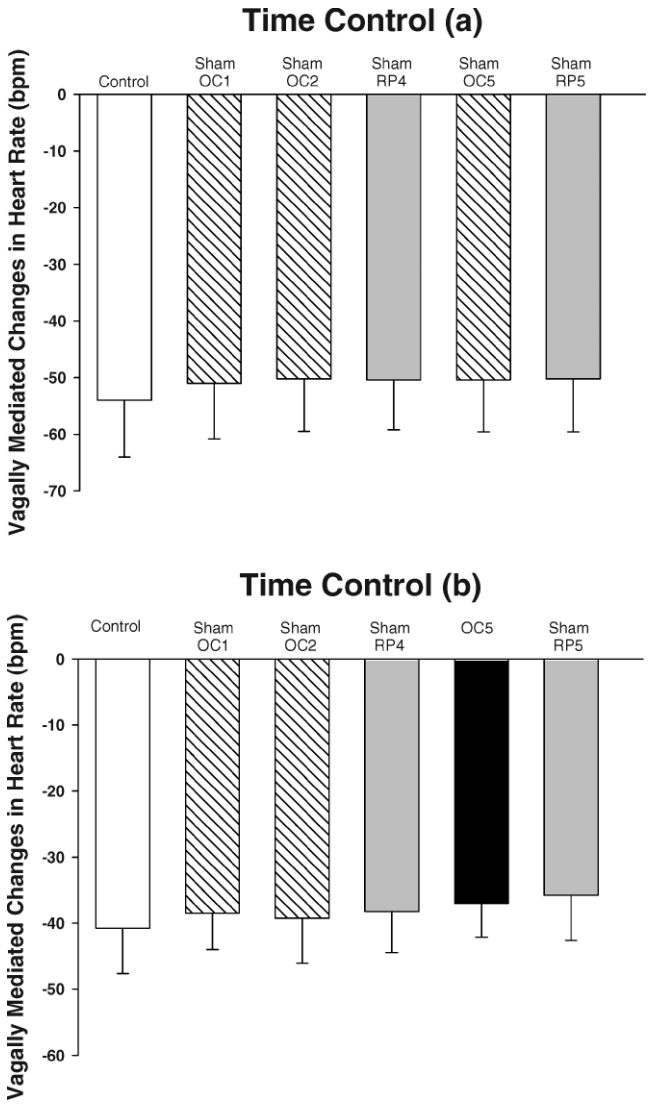

Figure 3. Changes in heart rate mediated by repeated right vagus nerve stimulation are illustrated for sham occlusions. Stimulation intervals correspond to the periods of occlusion and reperfusion in Figure 2. Figure 3b includes a single occlusion corresponding to occlusion five. Values are means and standard error of the mean for five and four subjects, respectively.

nodal artery was occluded for a 6th time and MEAP was added back to the dialysis inflow, the inhibitory effect on vagal stimulation was reduced further from 21% to 13% of the original control. In order to determine whether the perceived loss of efficacy during occlusion was specific to the occlusion, the MEAP was continued, the slipknot was released and the vagus was reevaluated 10 mins later. Surprisingly, the vagolytic response of MEAP was reduced further yet from 13% to 0% and the rate of attrition seemed to have accelerated. The MEAP was discontinued and the restoration of the vagal response was demonstrated after 10 mins. The percent changes were smaller if calculated from the post-PC washout; however, the progressive pattern of attrition was identical.

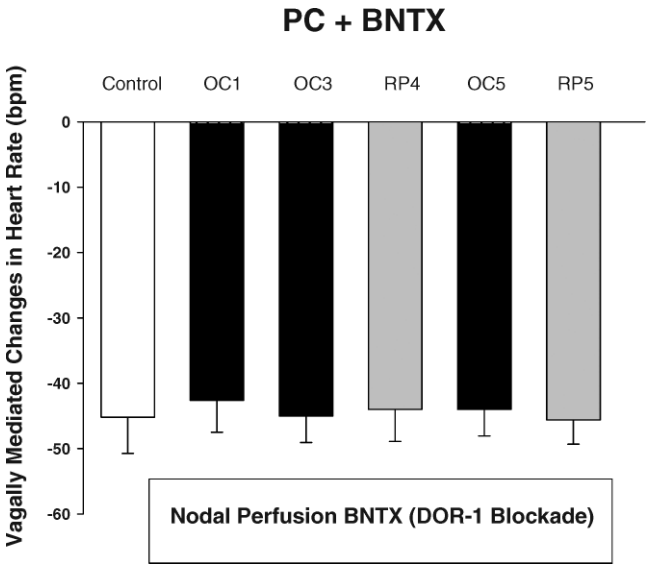

Figure 4. The figure illustrates the effect of DOR-1 blockade with BNTX on changes in heart rate mediated by right vagal nerve stimulation during the PC protocol. Values are means and standard error of the mean for five subjects.

Study 2(b) Sham IPC Protocol and Exogenous MEAP. Figures 6a and 6b illustrate the results of the MEAP evaluations following the sham-PC protocols in the presence and absence of occlusions during OC5. In the absence of

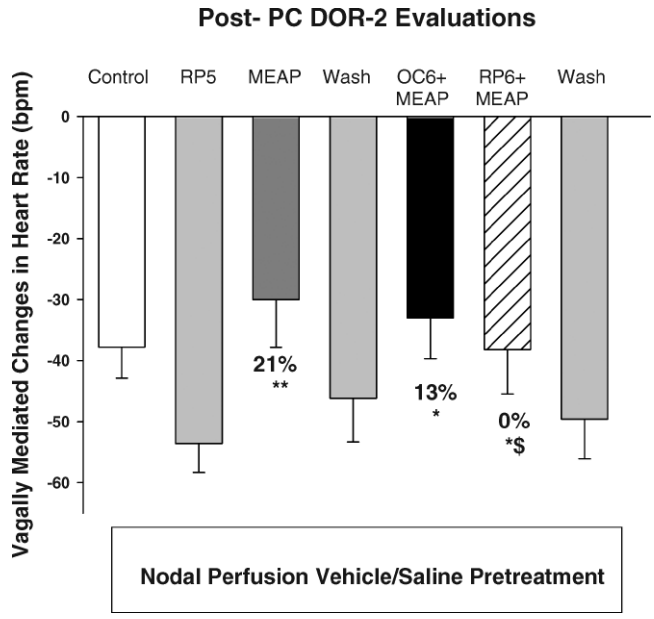

Figure 5. Figure 5 illustrates the effects of MEAP administered by microdialysis into the SA node interstitium following completion of the PC protocol in Figure 2. The resulting changes in heart rate during right vagal stimulation are illustrated sequentially for MEAP combined with reperfusion, occlusion and reperfusion. Values are means and standard error of the mean for five subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P < 0.05$ and $P < 0.01$, respectively. The symbol ($) indicates the change in the heart rate was significantly different from MEAP ($P < 0.05$).

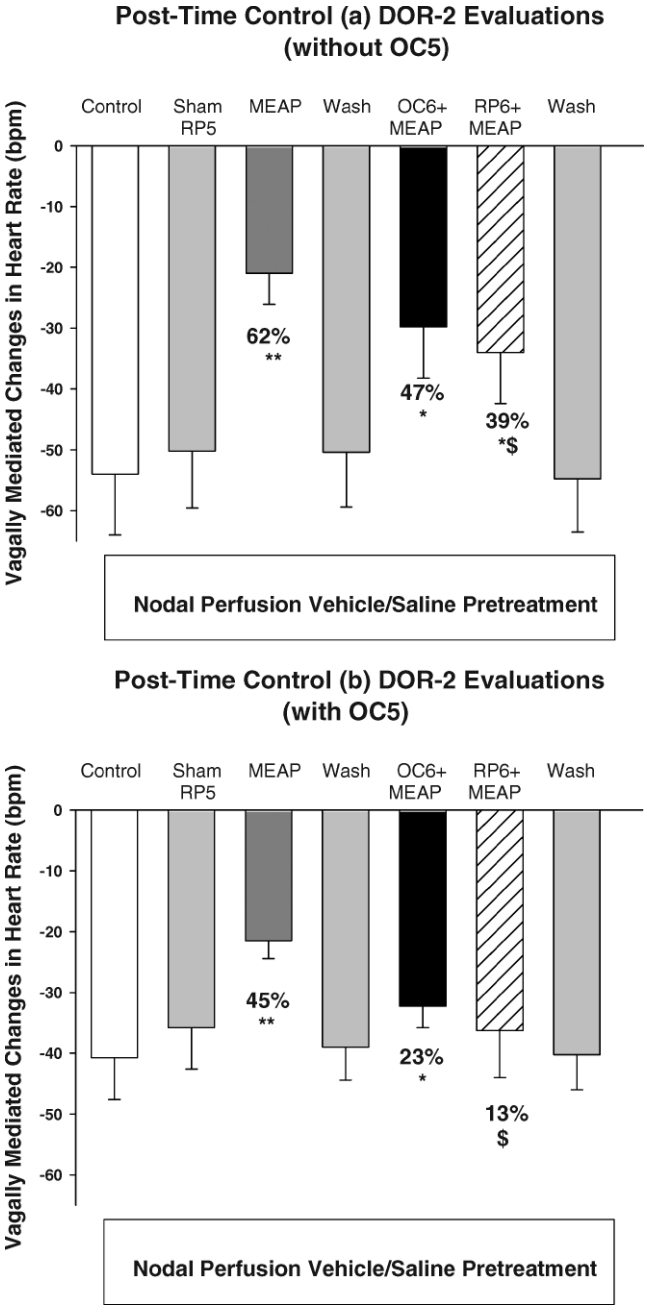

Figure 6. The figures illustrate the effects of MEAP administered by microdialysis into the SA node interstitium following completion of the sham PC protocols in Figure 3a and 3b. The resulting changes in heart rate during right vagal stimulation are illustrated sequentially for MEAP combined with reperfusion, occlusion and reperfusion. Values are means and standard error of the mean for five and four subjects, respectively. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P < 0.05$ and $P < 0.01$, respectively. The symbol ($) indicates the change in the heart rate was significantly different from MEAP at $P < 0.05$.

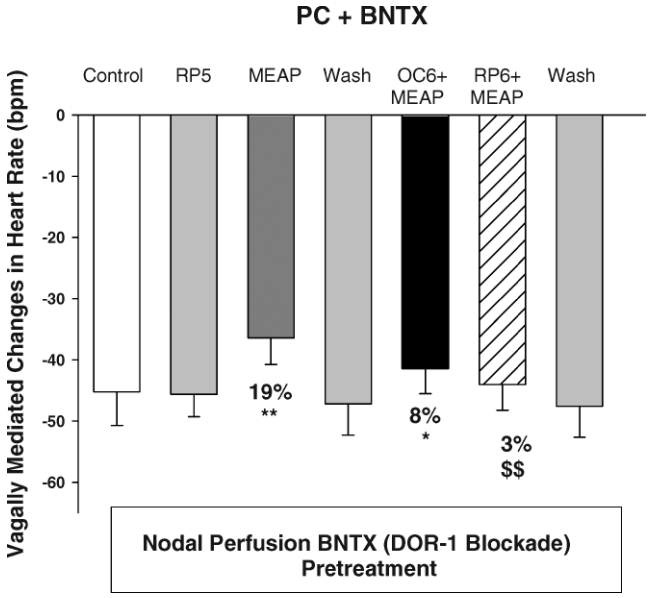

Figure 7. Figure 7 illustrates the effects of DOR-1 blockade with BNTX on MEAP administered by microdialysis into the SA node interstitium following completion of the PC protocol in Figure 4. The resulting changes in heart rate during right vagal stimulation are illustrated sequentially for MEAP and BNTX combined with reperfusion, occlusion and reperfusion. Values are means and standard error of the mean for five subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P < 0.05$ and $P < 0.01$, respectively. The symbol ($$) indicates the change in the heart rate during RP6 + MEAP was significantly different from MEAP administered after RP5 ($P < 0.01$).

prior PC occlusions, MEAP reduced the vagal bradycardia by 62% similar to the historical effect of MEAP in non-occluded animals. The vagal response was fully restored following washout. When the MEAP was reintroduced in combination with an initial occlusion, the magnitude of the vagolytic effect was again reduced, in this case from 62% to 47%. The MEAP was continued and the nodal artery occlusion was released. The subsequent vagal stimulation after 10 mins reperfusion with continued MEAP revealed a continued erosion of the vagolytic response from 47% to 39%. Once again washout fully restored the control vagal response. Although the declining vagolytic effects were less complete following the sham-PC protocol, the pattern of attrition was very similar to that after PC.

In order to evaluate the effects of prior occlusion on the vagolytic response of MEAP, the post-PC protocol was also conducted in the 5th occlusion shams. Following the single occlusion at interval five, MEAP reduced the vagal bradycardia by 45% as compared to 62% in the absence of that one prior occlusion. The vagolytic effect of MEAP declined still further to 23% during the subsequent (second) occlusion suggesting an interaction between the number of occlusions and MEAP. Following the release of the occlusion temporally equivalent to RP6, the MEAP response was reduced further (13% vs 39%) compared to the post-PC sham with only one occlusion. These observations support further an interaction between the number of occlusions and MEAP to down regulate the DOR-2 response.

Study 2(c) PC, DOR-1 Blockade and MEAP. Figure 7 illustrates the results of the MEAP evaluations following the PC protocol in the presence of DOR-1

blockade throughout the protocol. The post-PC vagolytic effect of MEAP was very weak, similar to that observed after PC in the absence of BNTX. Following PC + BNTX, MEAP reduced the vagally mediated decline in heart rate by only 19%. This value was quite comparable to that observed after PC alone (21%). Washing out the MEAP once again restored the vagally mediated bradycardia to control conditions. Reintroducing MEAP during nodal artery occlusion reduced the vagolytic influence of MEAP from 19% to 8%. When rechecked again 10 mins later during reperfusion, the vagolytic influence of MEAP was nearly eliminated at 3%. After washing out the MEAP, vagal stimulation produced a decline in heart rate that was again not different from control.

Study 2(d) Vagolytic Effects, Cross Study Comparisons. The initial 62% inhibition of vagally mediated bradycardia by MEAP in the sham was consistent with the 60–75% inhibition typically observed (6, 9, 11, 14–17, 21) indicating that time or repeated stimulation does not erode the vagolytic response. The 21–34% inhibition by MEAP observed after completing the PC protocol suggests that MEAP was now less effective compared with the Sham-PC (21% vs 62%, $P < 0.05$). MEAP remained inhibitory when administered during the occlusion in all three studies though the magnitude of that effect was less in the two PC studies during occlusion (47% vs 13% vs 8%, $P < 0.05$) and again during the subsequent reperfusion (39% vs 0% vs 3%, $P < 0.05$). Occlusion itself also may reduce the efficacy of MEAP.

Discussion

Individuals with strong parasympathetic control of the heart generally have better prognoses following adverse cardiovascular events. Those who recover vagally mediated heart rate variability quickly after myocardial infarction are much more likely to survive (23). Carotid massage acutely increases efferent parasympathetic traffic and is routinely employed to modify selected cardiac arrhythmias. Exercise training chronically increases parasympathetic influences on heart rhythms and reduces both myocardial damage and the incidence of ventricular fibrillation after coronary occlusion (3–5, 12, 13). In contrast, ventricular parasympathetic activity can extend the refractory period and facilitate the genesis of abnormal rhythms (42). Thus, the nuances of vagal transmission and the factors that modulate its plasticity are important targets for investigation.

Cardiac enkephalins (19, 25, 36) and the associated DORs are capable of dramatically modifying parasympathetic transmission at the vagal, myocardial interface. Although the opioids are traditionally viewed as exerting their influence by inhibition of neurotransmitter release, excitatory effects of opioids are routinely observed in many systems (22, 31). Nanomolar doses of MEAP administered by dialysis into the interstitium of the SA node interrupted vagal transmission. The DOR-2 antagonist naltriben selectively blocked that vagolytic influence. Femtomolar doses of MEAP in contrast increased the efficacy of vagal stimulation (17). Applying a PC protocol to the nodal artery produced similar femtomolar increases in MEAP in the nodal interstitium (21). An improved vagal transmission was observed afterward only during subsequent occlusions when MEAP was elevated. Both of these vagotonic effects were abrogated by sub-femtomolar infusions of the DOR-1 antagonist BNTX. The original four-cycle PC studies did not test whether the late occlusion alone, the prior PC protocol or both were both required to demonstrate the vagotonic effect.

Aim 1, Repeated Occlusion Improves the DOR-1 Response. The results obtained in the current study suggest that occlusion alone was not sufficient to produce an immediate vagotonic effect since no change in vagal efficacy was observed during the 1st occlusion. Graded improvements in vagal transmission were observed during the 3rd and 5th occlusions suggesting that the capability evolves over time. Whether the vagotonic response requires multiple occlusions or simply a single trigger occlusion to initiate the process followed by sufficient time for the vagotonic response to evolve remains unclear. However, the late occlusion performed during sham experiments clearly demonstrated that there was no improvement in the vagal function following an extended protocol and a single late occlusion.

The prior pharmacologic studies of DOR interactions in the canine SA node have suggested that the proportion of functioning DOR-1 and DOR-2 phenotypes can be fluid (9, 11) and the sum of their opposing influences are proposed to determine the net effect observed at any point in time (7). The PC mediated emergence of the vagotonic phenotype during the later occlusions in the PC protocol might thus represent an increase in DOR-1 influence and/or a reduction in the DOR-2 influence. The emerging vagotonic effect was clearly DOR-1 mediated throughout the five-occlusion protocol since DOR-1 blockade with BNTX eliminated the improved vagal transmission.

Aim 2, Repeated Occlusion Facilitates the Loss of DOR-2 Responses. The PC protocol also reduced the DOR-2 mediated vagolytic influence of added MEAP during both perfusion and occlusion. Historically, MEAP inhibits vagal transmission by 60–75% as observed in the time controls from this study. The initial 21–34% inhibition of vagal transmission observed after PC was lower than the expected 60–80% observed historically (15–16) and in this study in time controls during normal perfusion. The vagolytic effect of added MEAP declined progressively during the subsequent occlusion and reperfusion nearly disappearing during the final evaluation. The PC protocol clearly contributes to the DOR-2 decline which was much less complete in time controls. MEAP alone did not dramatically reduce its own vagolytic response even when repeatedly applied for two hours during normal perfusion (14). Thus, the decline in the DOR-2 response appears to be

more complicated than simple homologous desensitization. Repeated arterial occlusion appears to mediate a change in the receptor environment that favors agonist mediated down regulation of the DOR-2 response.

Summary. The studies discussed above sought to test the primary hypothesis that PC was responsible for the appearance of vagotonic effects. The second aim sought to test the hypothesis that PC reduced competing vagolytic effects. The analyses above suggest that both are correct; PC improves DOR-1 mediated vagotonic responses and accelerates the disappearance of DOR-2 mediated vagolytic responses. The hypothesis was formulated from previous studies that showed repeated SA node arterial occlusions raised endogenous opioids and produced corresponding improvement in the vagal function (17, 21). Whether these DOR mediated interactions with vagal transmission are an integral part of cardioprotective mechanisms or a convenient bioassay of DOR status remains open for discussion. Very little is known about the preconditioning effects on the right side of the heart or whether the changes observed in the SA node generalize to the remainder of the heart. However, a functional shift of DOR-2 to DOR-1 mediated responses would presumably be beneficial since the DOR-1 is the putative cardioprotective phenotype (24, 33, 34).

Aim 3, DOR-1 Blockade Accelerates DOR-2 Losses. The third experiment was designed to verify the DOR-1 character of the evolving vagotonic response and to test the hypothesis that PC mediated DOR-1 stimulation was responsible for the subsequent erosion and elimination of the DOR-2 vagolytic effect. BNTX completely prevented the PC mediated vagotonic effect indicating its mediation by DOR-1 receptors. This dose of BNTX was previously determined as ineffective against DOR-2 mediated responses (17). Contrary to the hypothesis, the subsequent challenges with added MEAP produced very weak vagolytic responses that quickly disappeared during sequential exposures to the same agonist. The rate of attrition in the response was nearly identical to the pattern observed after PC alone. PC appears to facilitate the loss of the vagolytic response perhaps independently of DOR-1 receptor stimulation. This is inconsistent with prior results in which DOR-1 stimulation with the selective DOR-1 agonist TAN-67 increased the rate of decline in DOR-2 responses (11). These conflicting observations would be compatible if the extended exposure to BNTX alters receptor trafficking (2, 8, 38). Thus, the PC mediated vagotonic response itself is DOR-1 mediated but the role of that DOR-1 stimulation in the subsequent PC mediated DOR-2 decline remains unresolved.

Sympathetic Participation. One might reasonably attribute increases and decreases in vagal influence to compensatory adjustments in the reciprocal activity of sympathetic innervation. Although sympathetic participation does not influence the specific questions addressed by the current study, a sympathetic role in the underlying mechanism is difficult to rule out completely. However,

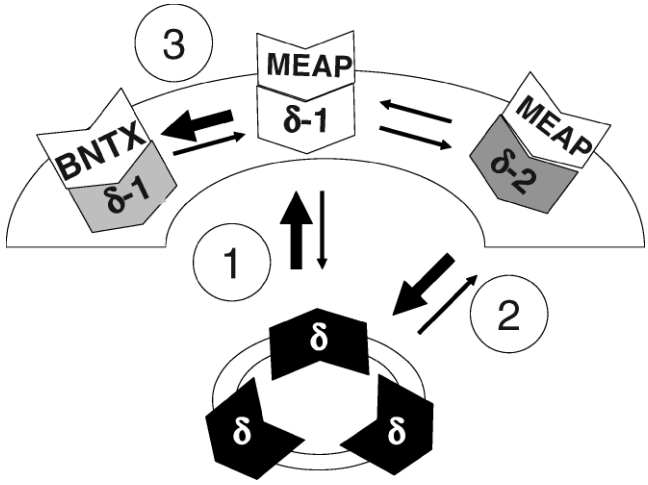

Figure 8. Figure 8 illustrates the proposed relationship between indifferent DORs within the sublemmal pool and DOR-1 and DOR-2 phenotypes on the cell surface.

several factors suggest that the observations reported here are the results of direct opioid/vagal adjustments. First, the vagal evaluations were conducted during the immediate response to nerve stimulation when vagal effects predominate prior to the emergence of potential sympathetic compensation. The vagolytic effect of MEAP is unaltered by prior adrenergic blockade (6) and nodal MEAP has no influence on sympathetically mediated tachycardia (44). Similarly, NE did not reduce the vagolytic effect of intranodal enkephalin delivered by dialysis and thus could not explain the progressive erosion of the vagolytic response (45). Finally, the DORs in the SA node and right atria colocalize almost exclusively with parasympathetic nerve endings and their isolated synaptosomes (10).

Proposed Mechanisms. Integrating the current findings with prior work supports a unifying hypothesis that intermittent metabolic stress increases local myocardial enkephalin production that then easily activates the more sensitive DOR-1 receptors on nearby vagal processes (10, 15, 17). The DOR-1 receptor stimulates adenylylcyclase activity in the prejunctional terminals through a GM-1, $G_{S\alpha}$-dependent coupling mechanism that increases calcium influx, vesicular transport and acetylcholine release (7, 22, 31). DOR-1 mediated increases in protein kinase A (PKA) increase GM-1 synthesis. The increased plasma membrane GM-1 completes a positive feedback loop by recruiting indifferent DORs into the DOR-1 phenotype as they emerge into the plasma membrane from the sublemmal compartment, #1 Figure 8. Thus, GM-1 increases the probability that emerging DORs assume the DOR-1 configuration. The hypothesis proposes further that $G_{i\alpha}$ and the inhibition of adenylylcyclase activity mediate the vagolytic action of DOR-2 stimulation (14, 26, 31). DOR-2 stimulation also dramatically increases the exchange of receptor between surface and cytoplasm, #2 Figure 8 (2, 8–10, 38). When GM-1 is available, the DOR-2 stimulated trafficking of

receptors into the cytoplasm of the nerve terminal favors the exchange of existing DOR-2 receptors for newly emerging DOR-1 receptors. This is consistent with both the DOR-1 and GM-1 mediated erosion of DOR-2 responses reported previously (9, 11).

The loss of the DOR-2 response following extended exposure to BNTX would be consistent with the trafficking hypothesis if BNTX sequesters the emerging receptors on the surface and removes them from the exchangeable circulating pool, #3 Figure 8. The declining pool of receptors would quickly deplete those available for either phenotype. Thus, the extended DOR-1 blockade eliminates all vagotonic influences directly and rapidly depletes the recycling pool of indifferent receptors available to mediate DOR-2 vagolytic responses. The relatively slow time course for the DOR-2 erosion during BNTX is far more compatible with down-regulation than competitive inhibition. A similar BNTX mediated loss of ganglionic DOR-2 responses occurred during the evaluation of enkephalin effects on hindlimb blood flow (43) suggesting a commonality among cholinergic junctions.

Experimental cardiac preconditioning often employs transient arterial occlusions or added opioids active at the DOR-1 receptor. Thus, understanding how to manipulate the proportion of DOR-1 and DOR-2 receptors could be valuable for inducing a cardioprotective phenotype with a favorable mixture of DORs.

In conclusion, the vagotonic result observed during arterial occlusion requires preconditioning for complete expression and could result in part from reduced competition from opposing vagolytic effects. BNTX abolished the vagotonic effects of preconditioning confirming DOR-1 participation. BNTX, however, failed to prevent the PC mediated erosion of the DOR-2 vagolytic responses. This conflicts with earlier observations that DOR-1 stimulation reduces DOR-2 responses (9, 11) and supports the suggestion that BNTX alters DOR trafficking independent of its blockade of DOR-1 signaling.

Significance. These rapid changes in apparent DOR phenotypes represent a potentially important clinical target for modifying autonomic balance. DOR-1 predominance combined with an improved vagal transmission should represent an inherently cardioprotective phenotype. The rapid plasticity of these responses suggests the real prospect that one might be able to moderate the system for instance behaviorally through diet, physiologically through exercise or pharmacologically through designer opioids.

1. Allouche S, Hasbi A, Ferey V, Sola B, Jauzak P, Polastron J. Pharmacological δ-1 and δ-2 opioid receptor subtypes in the human neuroblastoma cell line SK-N-BE: no evidence for distinct molecular entities. Biochem Pharmacol 59:915–925, 2000.
2. Bao L, Jin SX, Zhang C, Wang LH, Xu ZZ, Zhang FX, Wang LC, Ning FS, Cai HJ, Guan JS, Xiao HS, Xu ZQ, He C, Hökfelt T, Zhou Z, Zhang X. Activation of delta opioid receptors induces receptor insertion and neuropeptide secretion. Neuron 37(1):121–33, 2003.
3. Billman GE, Schwartz PJ, Stone HL. The effects of daily exercise on susceptibility to sudden cardiac death. Circulation 69(6):1182–1189, 1984.
4. Binkley PF, Nunziata E, Haas GJ, Nelson SD, Cody RJ. Parasympathetic withdrawal is an integral component of autonomic imbalance in congestive heart failure: demonstration in human subjects and verification in a paced canine model of ventricular failure. J Am Coll Cardiol 18(2):464–472, 1991.
5. Brown DA, Moore RL. Perspectives in innate and acquired cardioprotection: cardioprotection acquired through exercise. J Appl Physiol 103(5):1894–1899, 2007.
6. Caffrey JL, Mateo Z, Napier LD, Gaugl JF, Barron BA. Intrinsic cardiac enkephalins inhibit vagal bradycardia in the dog. Am J Physiol 268(2 Pt 2):H848–H855, 1995.
7. Crain SM, Shen KF. After chronic opioid exposure sensory neurons become supersensitive to the excitatory effects of opioid agonists and antagonists as occurs after acute elevation of GM1 ganglioside. Brain Res 575(1):13–24, 1992.
8. Cvejic S, Devi LA. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J Biol Chem 272(43):26959–26964, 1997.
9. Davis S, Deo SH, Barlow M, Yoshishige D, Farias M, Caffrey JL. The monosialosyl ganglioside GM-1 reduces the vagolytic efficacy of delta2-opioid receptor stimulation. Am J Physiol Heart Circ Physiol 291(5):H2318–H2326, 2006.
10. Deo SH, Barlow MA, Gonzalez L, Yoshishige Y, Caffrey JL. Cholinergic location of delta opioid receptors in canine atria and SA node. Am J Physiol Heart Circ Physiol 294:H829–H838, 2008.
11. Deo SH, Johnson-Davis S, Barlow MA, Yoshishige D, Caffrey JL. Repeated delta1-opioid receptor stimulation reduces delta2-opioid receptor responses in the SA node. Am J Physiol Heart Circ Physiol 291(5):H2246–H2254, 2006.
12. Dickson EW, Hogrefe CP, Ludwig PS, Ackermann LW, Stoll LL, Denning GM. Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. Am J Physiol Heart Circ Physiol 294:H402–H408, 2008.
13. Dickson EW, Ludwig PS, Ackermann LW, Buresh CT, Denning GM. Met5-enkephalin-Arg6-Phe7 (MEAP): a cardioprotective hormonal opioid. Acad Emerg Med 13:813–819, 2006.
14. Farias M 3rd, Jackson K, 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 285(5):H2001–H2012, 2003.
15. Farias M 3rd, Jackson KE, Yoshishige D, Caffrey JL. Cardiac enkephalins interrupt vagal bradycardia via delta 2-opioid receptors in sinoatrial node. Am J Physiol Heart Circ Physiol 284(5):H1693–H1701, 2003.
16. Farias M, Jackson K, Stanfill A, Caffrey JL. Local opiate receptors in the sinoatrial node moderate vagal bradycardia. Auton Neurosci 87(1): 9–15, 2001.
17. Farias M, Jackson K, Yoshishige D, Caffrey JL. Bimodal delta-opioid receptors regulate vagal bradycardia in canine sinoatrial node. Am J Physiol Heart Circ Physiol 285(3):H1332–H1339, 2003.
18. Gross GJ. Role of opioids in acute and delayed preconditioning. J Mol Cell Cardiol 35(7):709–718, 2003.
19. Howells RD, Kilpatrick DL, Bailey LC, Noe M, Udenfriend S. Proenkephalin mRNA in rat heart. Proc Natl Acad Sci U S A 83(6): 1960–1963, 1986.
20. Jackson KE, Farias M, Stanfill A, Caffrey JL. Delta opioid receptors inhibit vagal bradycardia in the sinoatrial node. J Cardiovasc Pharmacol Ther 6(4):385–393, 2001.
21. Jackson KE, Farias M, Stanfill AS, Caffrey JL. Transient arterial occlusion raises enkephalin in the canine sinoatrial node and improves vagal bradycardia. Auton Neurosci 94(1–2):84–92, 2001.
22. Keren O, Gafni M, Sarne Y. Opioids potentiate transmitter release from

SK-N-SH human neuroblastoma cells by modulating N-type calcium channels. Brain Res 764(1–2):277–282, 1997.

23. Kleiger RE, Miller JP, Bigger JT Jr, Moss AJ. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. Am J Cardiol 59(4):256–262, 1987.
24. Kuzume K, Wolff RA, Amakawa K, Kuzume K, Van Winkle DM. Sustained exogenous administration of Met5-enkephalin protects against infarction in vivo. Am J Physiol Heart Circ Physiol 285(6): H2463–H2470, 2003.
25. Lang RE, Hermann K, Dietz R, Gaida W, Ganten D, Kraft K, Unger T. Evidence for the presence of enkephalins in the heart. Life Sci 32(4): 399–406, 1983.
26. Loh HH, Smith AP. Molecular characterization of opioid receptors. Annu Rev Pharmacol Toxicol 30:123–147, 1990.
27. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74(5): 1124–1136, 1986.
28. Paradis P, Dumont M, Belichard P, Rouleau JL, Lemaire S, Brakier-Gingras L. Increased preproenkephalin A gene expression in the rat heart after induction of a myocardial infarction. Biochem Cell Biol 70(7):593–598, 1992.
29. Portoghese PS, Sultana M, Nagase H, Takemori AE. A highly selective delta 1-opioid receptor antagonist: 7-benzylidenenaltrexone. Eur J Pharmacol 218(1):195–196, 1992.
30. Przyklenk K, Darling CE, Dickson EW, Whittaker P. Cardioprotection 'outside the box'—the evolving paradigm of remote preconditioning. Basic Res Cardiol 98(3):149–157, 2003.
31. 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 21(11):1353–1361, 1996.
32. Schallmach E, Steiner D, Vogel Z. Adenylyl cyclase type II activity is regulated by two different mechanisms: implications for acute and chronic opioid exposure. Neuropharmacology 50(8):998-1005, 2006.
33. Schultz JE, Hsu AK, Gross GJ. Ischemic preconditioning in the intact rat heart is mediated by delta1- but not mu- or kappa-opioid receptors. Circulation 97(13):1282–1289, 1998.
34. Schultz JE, Hsu AK, Gross GJ. Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. Circ Res 78(6):1100–1104, 1996.
35. Schultz JE, Rose E, Yao Z, Gross GJ. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Am J Physiol 268(5 Pt 2):H2157–H2161, 1995.
36. Springhorn JP, Claycomb WC. Translation of heart preproenkephalin mRNA and secretion of enkephalin peptides from cultured cardiac myocytes. Am J Physiol 263(5 Pt 2):H1560–H1566, 1992.
37. Van Wylen DG, Willis J, Sodhi J, Weiss RJ, Lasley RD, Mentzer RM Jr. Cardiac microdialysis to estimate interstitial adenosine and coronary blood flow. Am J Physiol 258(6 Pt 2):H1642–H1649, 1990.
38. Whistler JL, Tsao P, von Zastrow M. A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes. J Biol Chem 276(36):34331–34338, 2001.
39. Yao Z, Gross GJ. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. Am J Physiol 264(6 Pt 2): H2221–H2225, 1993.
40. Younes A, Pepe S, Barron BA, Spurgeon HA, Lakatta EG, Caffrey JL. Cardiac synthesis, processing, and coronary release of enkephalin-related peptides. Am J Physiol Heart Circ Physiol 279(4):H1989–H1998, 2000.
41. Younes A, Pepe S, Yoshishige D, Caffrey JL, Lakatta EG. Ischemic preconditioning increases the bioavailability of cardiac enkephalins. Am J Physiol Heart Circ Physiol 289(4):H1652–H1661, 2005.
42. Warner MR, Zipes DP. Vagal control of myocardial refractoriness. In: Levy MN, Schwartz PJ, Eds. Vagal Control of the Heart. Armonk, New York: Futura Publishing Co., pp261–273, 1994.
43. Barlow MA, Deo SH, Gonzalez L, Caffrey JL. Delta receptor phenotypes and vascular conductance in skeletal muscle. FASEB J 21:957.7, 2007 (Abstract).
44. Barlow MA, Deo S, Johnson-Davis S, Caffrey JL. Vagotonic effects of enkephalin are not mediated by sympatholytic mechanisms. Exp Biol Med 231:387–395, 2006.
45. Stanfill, AA, Jackson KE, Farias M, Barlow M, Deo, S, Johnson S, JL Caffrey. Leucine-enkephalin interrupts sympathetically mediated tachycardia prejunctionally in the canine sinoatrial node. Exp Biol Med 228:898–906, 2003.
46. Peart, JN, Patel HH, Gross GJ. Delta-opioid receptor activation mimics ischemic preconditioning in the canine heart. J Cardiovasc Pharmacol 42:78–81, 2003.