CHAPTER 3

Results

3.1 The effect of VNS on the electrocardiographic parameters during the I/R period

Figure 3-1.1a shows examples of ECG tracing at baseline, a marked elevation of the ST-segment after 5 minutes of LAD occlusion and ECG tracing returned to baseline after reperfusion. The electrophysiological effects of VNS were examined in 30 pigs in which heart rate (HR), PR interval, QRS duration, QT interval, T-wave peak to end (Tpe), and T-wave peak to end per QT interval ratio (Tpe/QT ratio) were continuously measured during the I/R period. In the I/R group the HR during the ischemic period increased significantly when compared with the baseline and returned to the baseline after reperfusion (Figure 3-1.1b). HR at the baseline, during ischemia, and reperfusion periods was not different in VNS-treated groups. Interestingly, the HR significantly increased in the LtVNX group during ischemia whereas the significantly increased in HR during reperfusion period was observed in the RtVNX. However, in the atropine group, HR during ischemic and reperfusion periods significantly increased when compared with baseline. PR interval was no significant difference among all groups at the baseline, ischemic, and reperfusion periods (Figures 3-1.1c). In contrast, PR interval in the atropine group significantly decreased PR during ischemic and reperfusion periods when compared with baseline. QRS duration was not significant difference among all groups at the baseline, ischemic, and reperfusion periods (Figures 3-1.1d). Figure 3-1.2a shows Tpe duration at baseline and during ischemic and reperfusion periods in LC-VNS group. QT interval during ischemia and reperfusion were significantly decreased in all groups (Figure 3-1.2b). Tpe was significantly decreased in all VNS-treated groups, except the ischemic period in the RtVNX group and this effect was abolished by atropine (Figures 3-1.2c). Tpe/QT ratio in the I/R

group during the ischemic period increased significantly when compared with the baseline and that in the atropine group significantly increased in both ischemic and reperfusion periods. Interestingly, there was no significant difference in Tpe/QT ratio during the baseline, ischemic, and reperfusion periods in all VNS treated groups (Figure 3-1.2d).

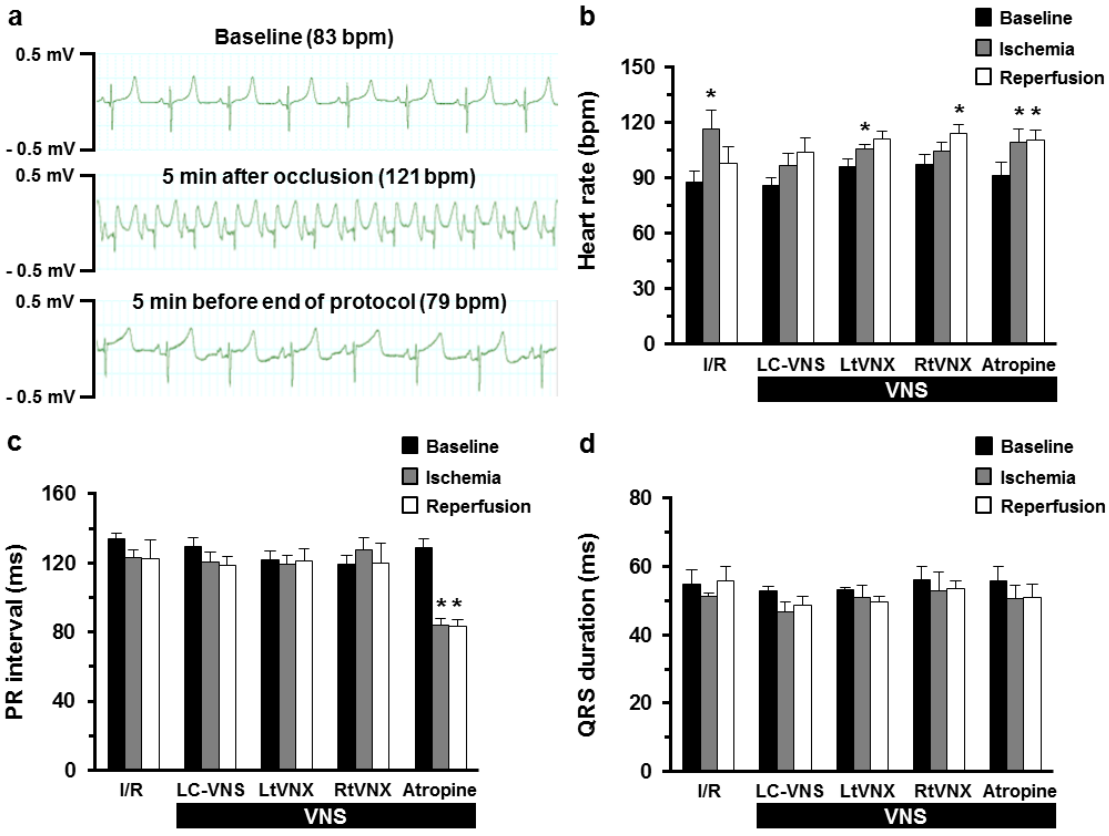

Figure 3-1.1: Effect of VNS on the electrocardiographic parameters during the ischemic and the reperfusion periods. a: Representative of the ECG baseline, 5 min after LAD ligation and 5 min before end of protocol. b: Effect of VNS on the heart rate. c: Effect of VNS on the mean PR interval. d: Effect of VNS on the mean QRS duration. Data are presented as mean ± SE. **P* < 0.05 vs baseline within group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection; VNS = vagus nerve stimulation.

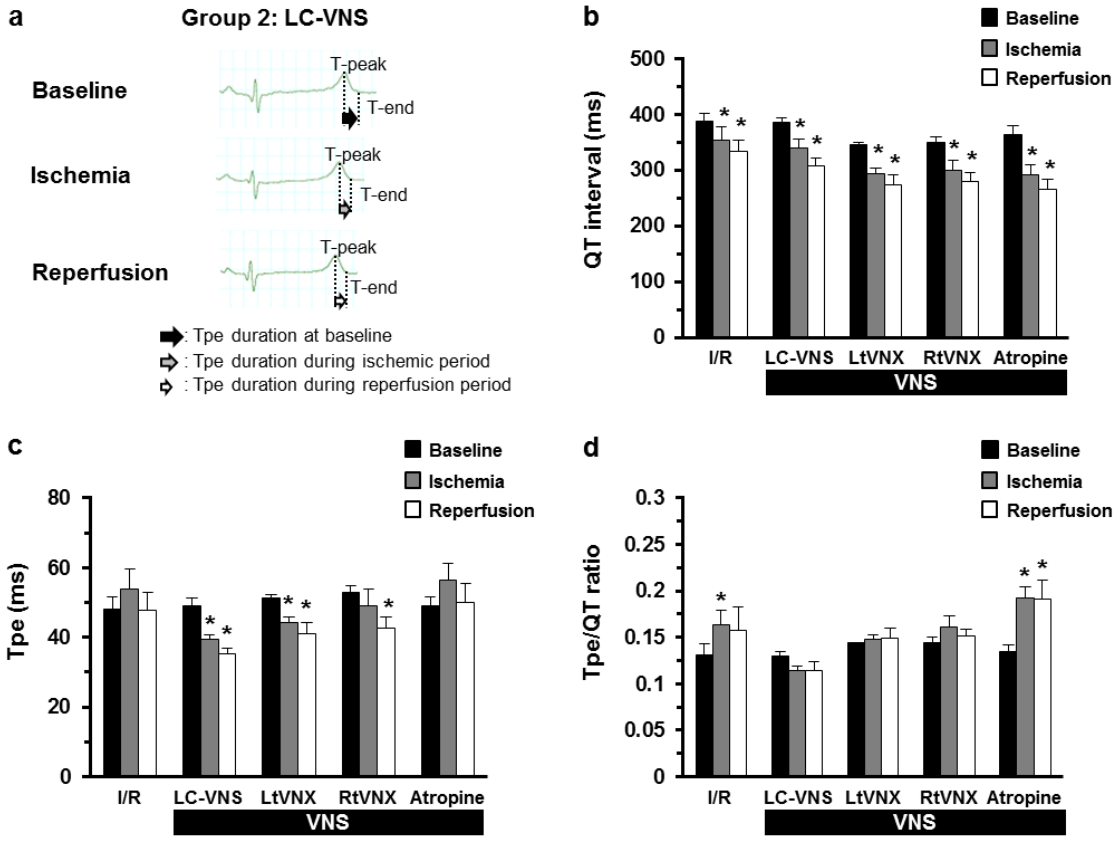

Figure 3-1.2: Effect of VNS on the electrocardiographic parameters during the ischemic and the reperfusion periods. a: Representative of the Tpe duration seen in LC-VNS group at baseline and during ischemic and reperfusion periods. b: Effect of VNS on the QT interval. c: Effect of VNS on the Tpe interval. d: Effect of VNS on the Tpe/QT ratio. Data are presented as mean ±SE. **P* < 0.05 vs baseline within group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; QT = QT interval; RtVNX = right vagus nerve transection; Tpe = T-wave peak to end; VNS = vagus nerve stimulation.

3.2 The effect of VNS on the occurrence of cardiac arrhythmia during the I/R period

Figure 3-2a represents a premature ventricular contractions (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF). The total number of PVC markedly decreased for both LC-VNS and LtVNX groups when compared with the I/R group (Figure 3-2b). However, the total number of PVC in the RtVNX group was not significantly decreased when compared with the I/R group. The result in the RtVNX group was similar to that of the atropine group. The total number of VT/VF episodes was significantly reduced in all VNS-treated groups compared with the I/R group (Figure 3-2c). However, time to 1st VT/VF onset was not significantly different among groups (Figure 3-2d). The arrhythmia score was significantly decreased in both LC-VNS and LtVNX groups when compared with I/R group (Figure 3-2e). Figure 3-2f shows the effect of VNS on connexin43 phosphorylation at serine 368. The connexin43 phosphorylation was significantly increased in both the LC-VNS group when compared with the I/R group. However, the levels of the phosphorylated connexin43 in the LtVNX and RtVNX groups were not significantly increased. This effect was abolished by the administration of atropine.

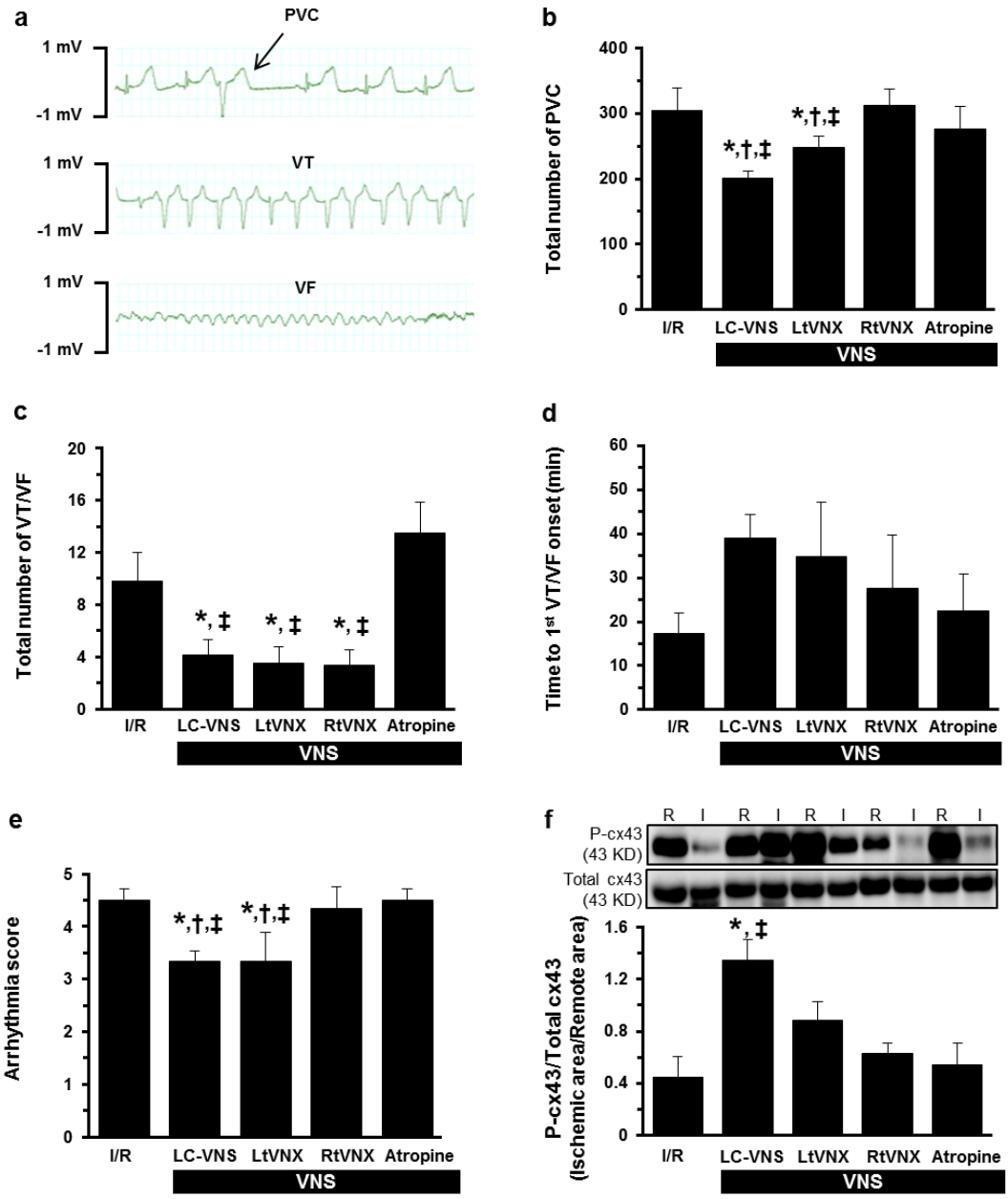

Figure 3-2: Effect of VNS on the occurrence of ventricular arrhythmias during the ischemic/reperfusion period. a: Representative of the PVC, VT and VF. b: Effect of VNS on the total number of PVC. c: Effect of VNS on the total number of VT/VF. d: Effect of VNS on the time to 1st VT/VF. e: Effect of VNS on the arrhythmia score. f: Effect of VNS on the phosphorylation of connexin43 at serine 368 in the ischemic myocardium. Data are presented as mean ± SE. *P < 0.05 vs I/R group; †P < 0.05 vs RtVNX group; ‡P < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left vagus nerve transection; PVC = premature ventricular contraction; VF = ventricular fibrillation; VNS = vagus nerve stimulation; VT = ventricular tachycardia.

3.3 The effect of VNS on LV function during the I/R period

The effect of VNS on LV functional performance is shown in figure 3-3. In the I/R group, the stroke volume (SV), ejection fraction (EF) and stroke work (SW) were significantly decreased and the end-diastolic pressure (EDP) was significantly increased during the ischemic and reperfusion periods when compared with the baseline period. Interestingly, all groups treated with VNS preserved LV functions during the ischemic and reperfusion periods. Moreover, all VNS treated groups significantly increased SV, EF and SW when compared with I/R and atropine groups in both ischemic and reperfusion periods. On the other hand, all groups treated with VNS (except LtVNX) significantly decreased EDP when compared with I/R and atropine groups in both ischemic and reperfusion periods. The beneficial effects of VNS were completely abolished by the administration of atropine. .

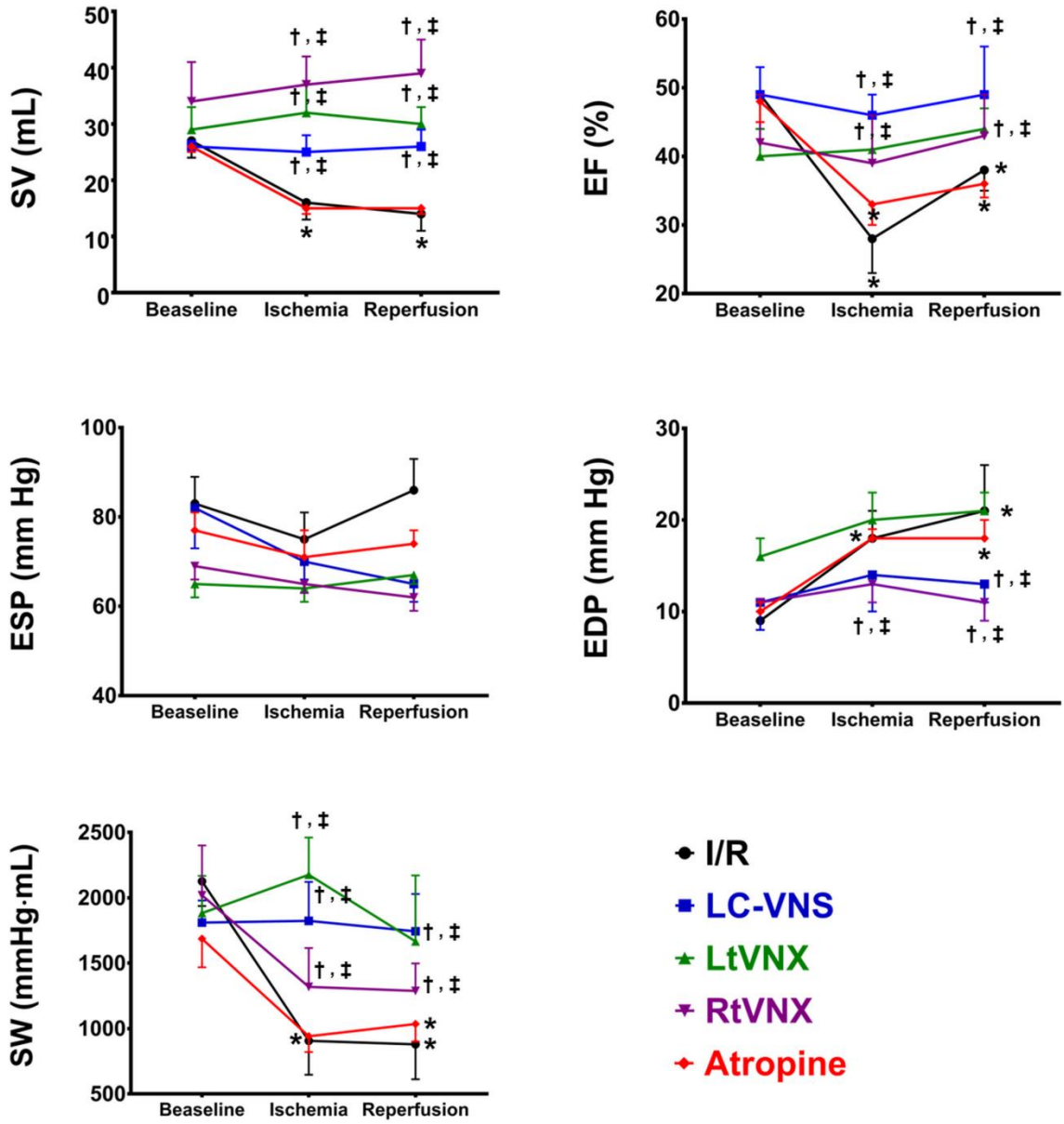

Figure 3-3: The effect of VNS on LV function at baseline, at 5 min before the end of ischemia, and at 5 min before the end of reperfusion (n = 6 per group). Values are presented as mean ± SE. **P* < 0.05 vs baseline within group; †*P* < 0.05 vs I/R group at that period; ‡*P* < 0.05 vs Atropine group at that period. EDP = end-diastolic pressure; EF = ejection fraction; ESP = end-systolic pressure; I/R = ischemic/reperfusion; LC-VNS = vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection; SV= stroke volume; SW = stroke work.

3.4 The effect of VNS on myocardial infarct size after the ischemic/reperfusion period

The percentage of the area at risk (AAR), which is expressed as a percentage of the total ventricular mass, was used to indicate myocardial infarct size. The AAR was not different among groups (I/R 32.8% ± 1.8%; LC-VNS 35.6% ± 2.4%; LtVNX 32.2% ± 1.7%; RtVNX 32.9 % ±0.8 % ; Atropine 38.1% ± 1.9% ; $P > 0.05$) (Figure 3-4a). All groups treated with VNS significantly reduced myocardial infarct size when compared with the I/R group and this effect was reversed by atropine. Interestingly, the myocardial infarct size was significantly increased in the RtVNX group when compared with those in the LC-VNS and in the LtVNX groups. In contrast, the infarct sizes was not different between those in the LC-VNS and in the LtVNX group (I/R 45.7% ± 5.4%; VNS 5.1% ± 1.3%; LtVNX 7.1% ± 1.8%; RtVNX 17.6% ± 1.4%; Atropine 52.9 % ± 3.6 %; $P < 0.05$) (Figure 3-4b). Figure 3-4c demonstrates representative Evan Blue and triphenyltetrazolium chloride (TTC) staining of heart sections to show the infarct area after I/R (white).

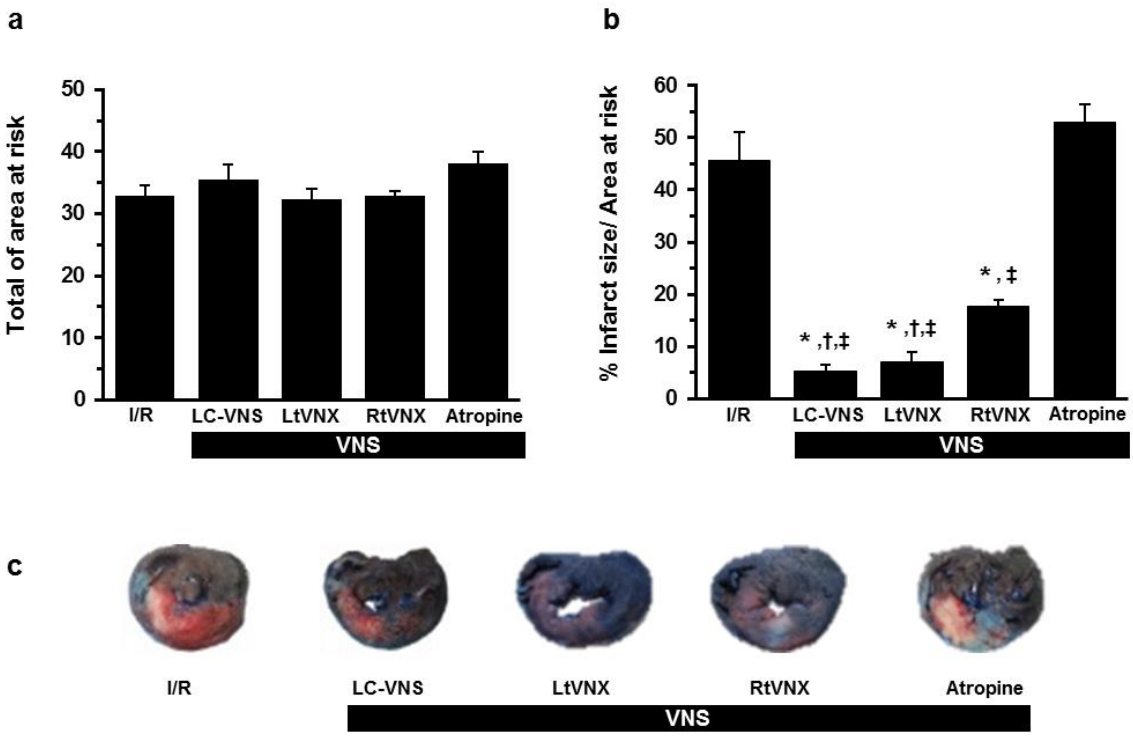

Figure 3-4: Effect of VNS on infarct size. a: Myocardial infarct size was expressed as the percentage of the area at risk (AAR). b: Effect of VNS on the myocardial infarct size. c: Representative Evan Blue and triphenyltetrazolium chloride (TTC) staining of heart sections. Blue indicates the non-threatened myocardium, red indicates the non-infarcted area within the AAR, and white indicates myocardial infarction. Data are presented as mean ± SE. **P* < 0.05 vs I/R group; †*P* < 0.05 vs RtVNX group; ‡*P* < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.5 The effect of VNS on cardiomyocyte apoptosis

TUNEL staining was performed to detect cardiomyocyte apoptosis. TUNEL positive cells, reported as the percentage of total nuclei, were significantly increased in ischemic area when compared with remote area in the I/R injury group. There was significantly decreased in all groups treated with VNS. Interestingly percentage of TUNEL positive cells per total nuclei was significantly increased in the RtVNX group when compared with those in the LC-VNS and in the LtVNX groups. Again, this effect was reversed by atropine (figure 3-5.1a). Figure 3-5.1b represents a TUNEL assay.

The cardioprotective effect of VNS was examined by the measurement of the key markers for apoptosis expression, which are Bax, cleaved caspase-3 (promoting apoptosis), and Bcl-2 (anti-apoptosis) (Figure 3-5.2). The expression of Bax and the Cleaved caspase-3/Pro caspase-3 ratio was significantly decreased in the LC-VNS, the LtVNX, and the RtVNX groups whereas the levels of Bcl-2 were significantly increased in the LC-VNS, the LtVNX groups, but not in the RtVNX, when compared with the I/R group. The administration of atropine totally abolished the cardioprotective effects of VNS with respect to cardiomyocyte apoptosis.

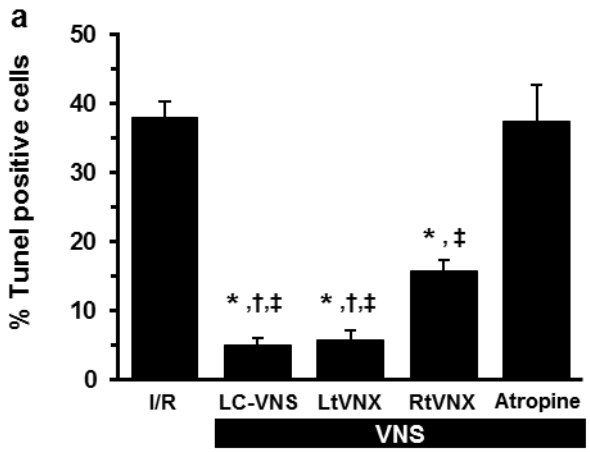

Figure 3-5.1a: Effect of VNS on the TUNEL positive cells, reported as the percentage of total nuclei. Data are presented as mean ± SE. *$P < 0.05$ vs I/R group; †$P < 0.05$ vs RtVNX group. ‡$P < 0.05$ vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

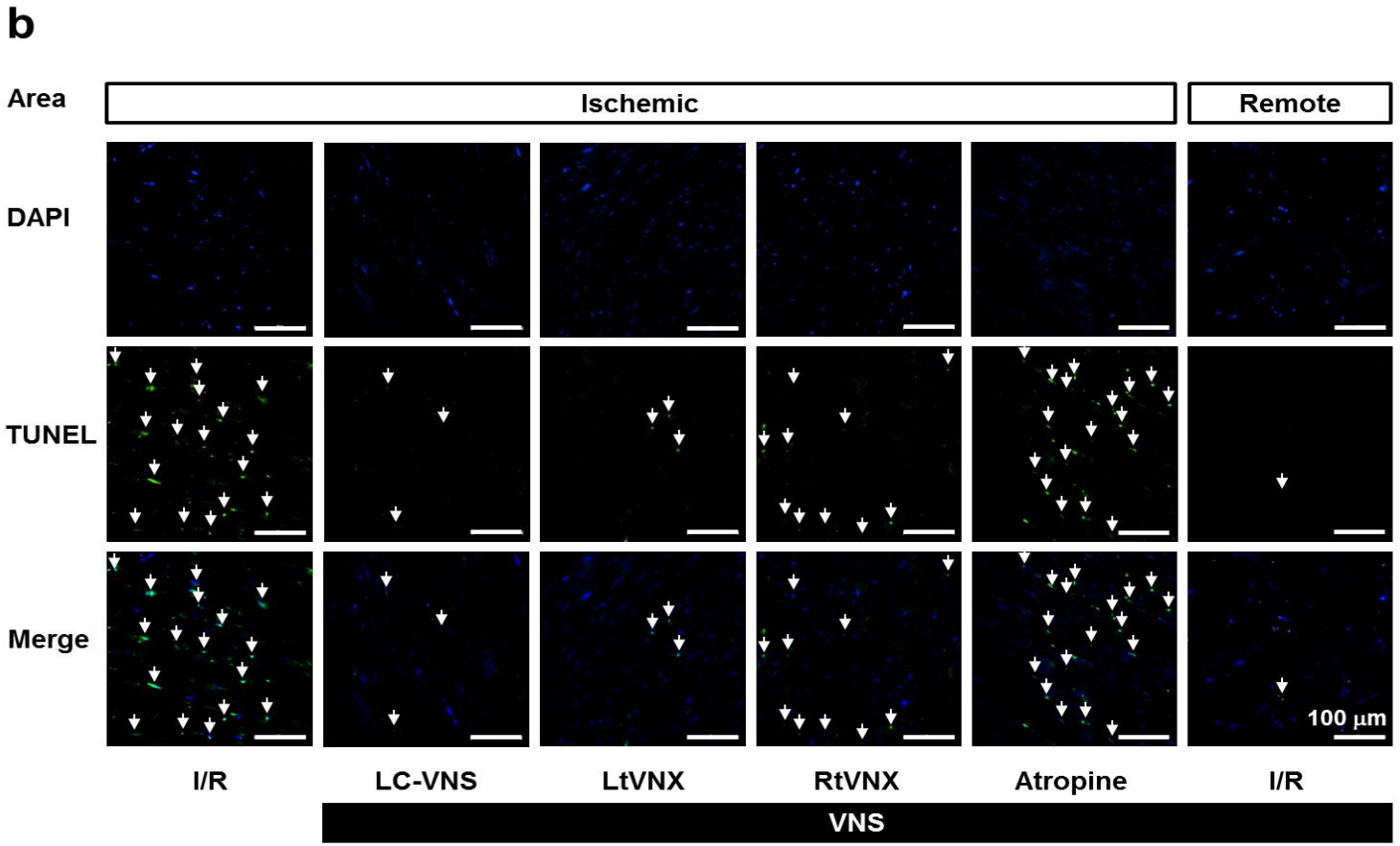

Figure 3-5.1b: Representative of the TUNEL assay. White arrow indicates TUNEL positive cells. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

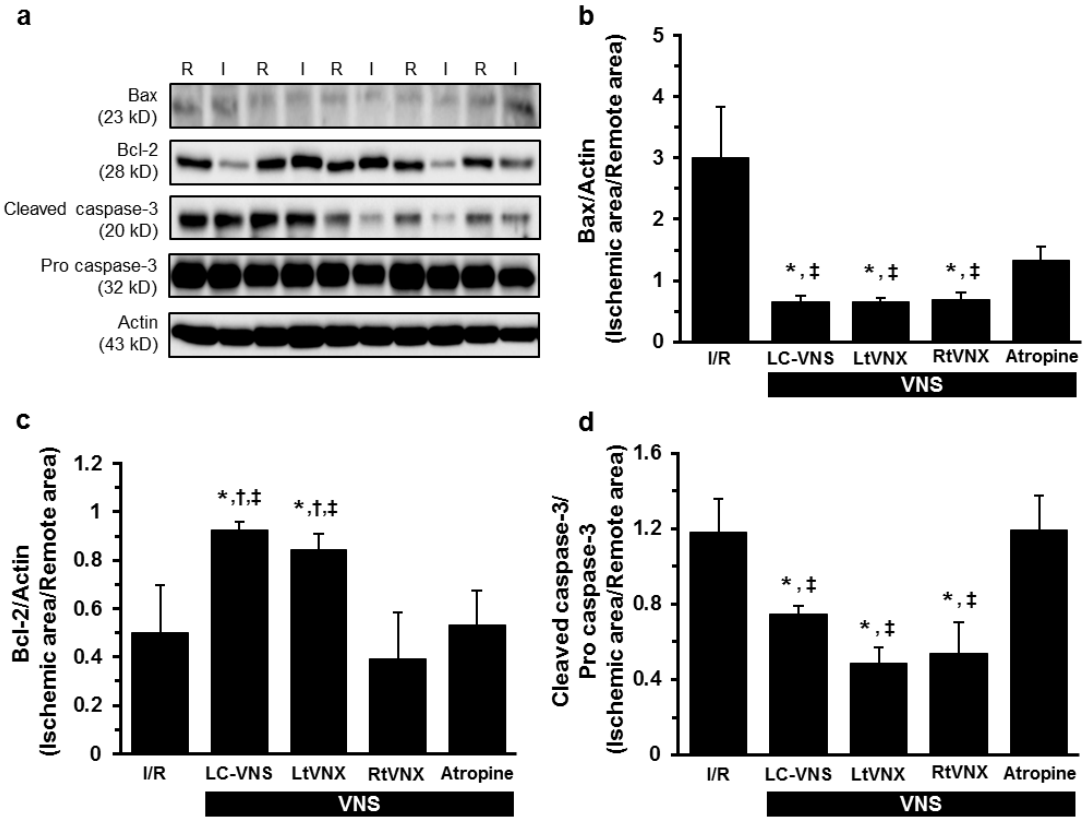

Figure 3-5.2: Effect of VNS on cardiomyocyte apoptosis. a: Specific bands at 23 kDa for Bax, at 28 kDa for Bcl-2, at 20 kDa for Cleave caspase-3 and at 32 kDa for Pro caspase-3 were detected in the ischemic and remote area among three groups. Antibodies for actin with a specific band at 43 kDa were used as reference. b and c: Western blot analysis of Bax and Bcl-2. d: Relative expressions are presented as the ratio between cleaved caspase-3 and Pro caspase-3 expression. Data are presented as mean ± SE. *$P < 0.05$ vs I/R group; †$P < 0.05$ vs RtVNX group; ‡$P < 0.05$ vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.6 The effect of VNS on oxidative stress activity (MDA)

Figure 3-6 shows the changes of MDA levels in myocardium between ischemic and remote areas. VNS treatment groups significantly decreased the level of MDA in the cardiac tissues when compared with the I/R group. However, there was a statistic difference in the RtVNX group when compared with the LC-VNS and LtVNX groups. This effect was abolished by the administration of atropine.

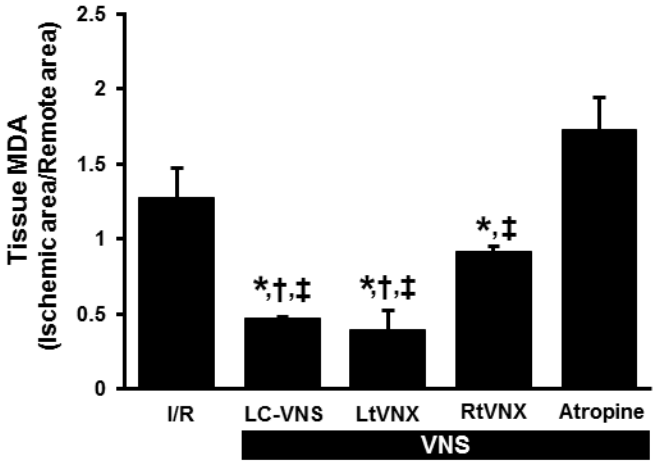

Figure 3-6: Effect of VNS on oxidative stress biomarker in myocardium. The changes of MDA levels in myocardium between ischemic and remote areas represent oxidative stress biomarker. Data are presented as mean ± SE. **P* < 0.05 vs I/R group; †P < 0.05 vs RtVNX group; ‡P < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.7 The effect of VNS on pro-inflammatory and anti-inflammatory markers

Figure 3-7a shows the changes of TNF-α levels in myocardium between ischemic and remote areas. LC-VNS group significantly decreased the levels of TNF-α in the cardiac tissues when compared with the I/R group. There was no a significant decrease in the level of TNF-α in the RtVNX, the LtVNX and the atropine groups when compared with the I/R group. Moreover, the levels of IL-10 in the myocardium were not significantly different in all groups (Figure 3-7b).

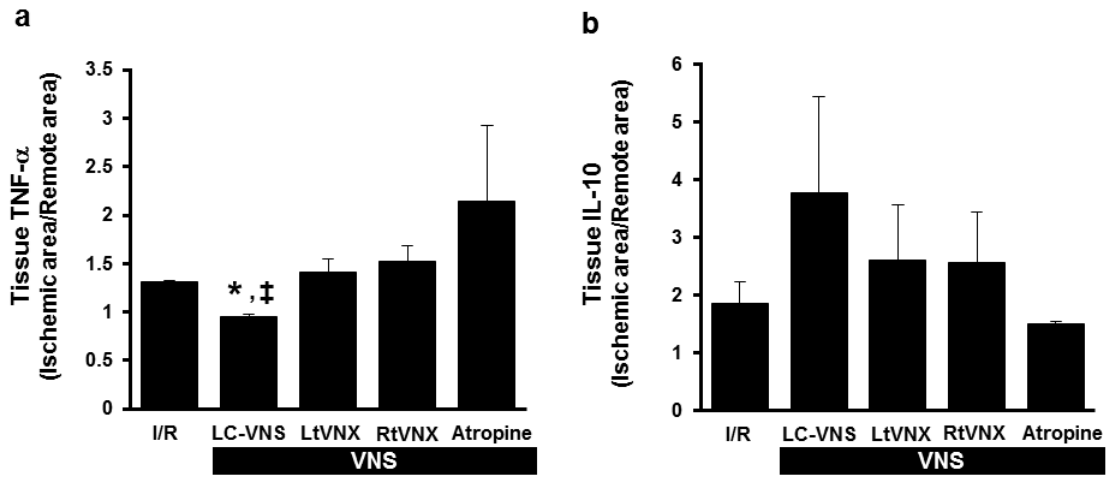

Figure 3-7: Effect of VNS on pro-inflammatory and anti-inflammatory markers. a: the changes of TNF-α levels in myocardium between ischemic and remote areas. b: the changes of IL-10 levels in myocardium between ischemic and remote areas. Data are presented as mean ± SE. *$P < 0.05$ vs I/R group; ‡$P < 0.05$ vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.8 The effect of VNS on mitochondrial function

An increase in reactive oxygen species (ROS) production, a decrease in red/green fluorescent intensity ratio (which indicated mitochondrial membrane depolarization), and a decrease in the absorbance (indicative of mitochondrial swelling) has been used to indicate cardiomyocyte-mitochodrial dysfunction. Both LC-VNS and LtVNX groups significantly decreased mitochondrial ROS production (Figure 3-8a) and prevented mitochondrial membrane depolarization (Figure 3-8b) and mitochondrial swelling (Figure 3-8c) in the ischemic myocardium as compared with the ischemic area of the I/R group. The RtVNX group significantly decreased mitochondrial ROS production and prevented mitochondrial membrane depolarization. However, the RtVNX group no significantly decreased mitochondrial swelling in the ischemic myocardium as compared with the ischemic area of the I/R group. Electron photomicrographs demonstrated that in the ischemic area of the I/R group, I/R-induced severe mitochondrial damage was observed, as demonstrated by noted mitochondrial swelling accompanied by a disruption in membrane integrity (Figure 3-8d). LC-VNS and LtVNX groups significantly attenuated cardiac mitochondrial swelling after I/R injury, but RtVNX group was no significantly attenuated cardiac mitochondrial swelling after I/R injury and this effect was abolished by atropine.

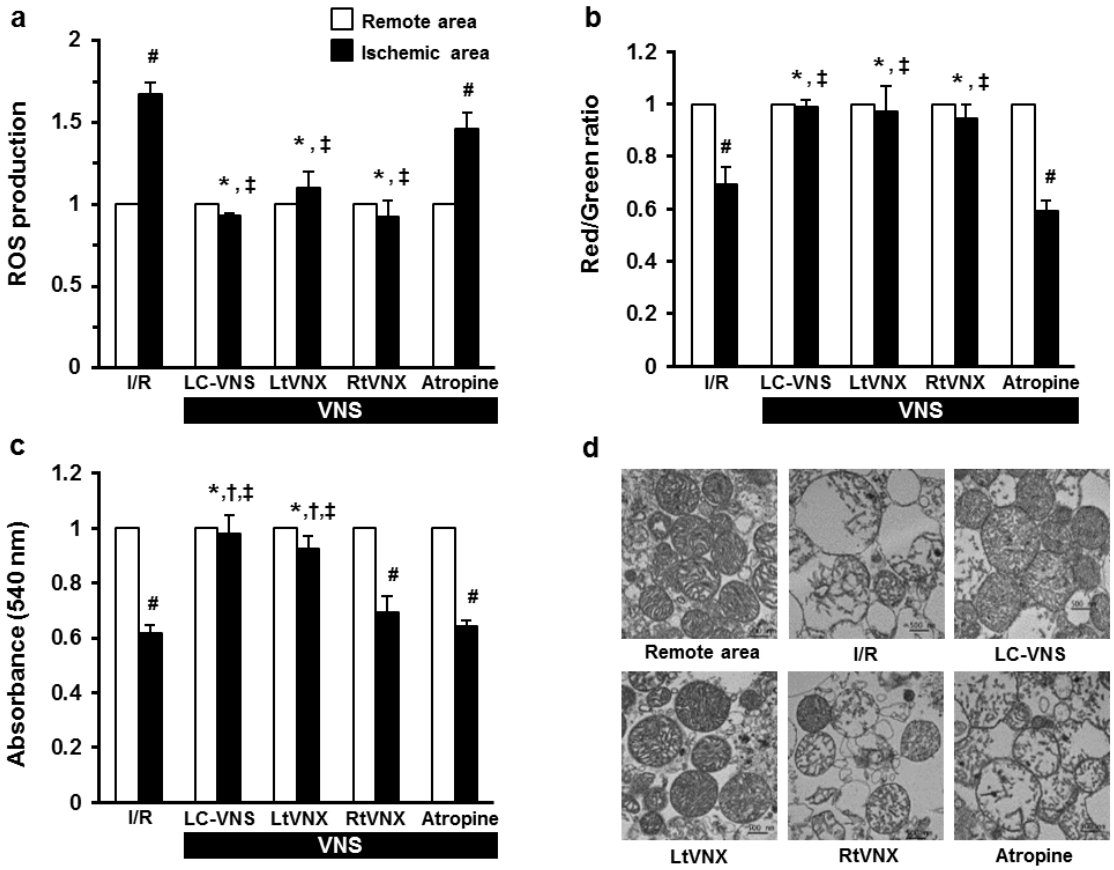

Figure 3-8: Effect of VNS on cardiac mitochondria function after ischemia and reperfusion periods. a: mitochondrial ROS production. b: mitochondrial membrane depolarization. c: mitochondrial swelling. d: Representative electron photomicrographs of a cardiac mitochondrial ultrastructure. Data are presented as mean ± SE. #$P < 0.05$ vs remote area within group; *$P < 0.05$ vs I/R group; †P < 0.05 vs RtVNX group; ‡$P < 0.05$ vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.9 The effect of VNS on mitochondrial dynamics

The proteins, MFN2, OPA1, and DRP1, which are involved in mitochondrial dynamics were determined (Figure 3-9). Both LC-VNS and LtVNX groups significantly increased the expression of MFN2 , OPA1, and the phosphorylation of DRP1 at Ser 637 as well as significantly decreased phosphorylation of DRP1 at Ser 616 when compared with the I/R group. The RtVNX group significantly increased OPA1 but not MFN2 when compared with I/R group and significantly decreased phosphorylation of DRP1 at Ser 616 as well as no significant increase in phosphorylation of DRP1 at Ser 637 when compared with I/R group. These protein levels were not significantly difference in the atropine group when compared with the I/R group.

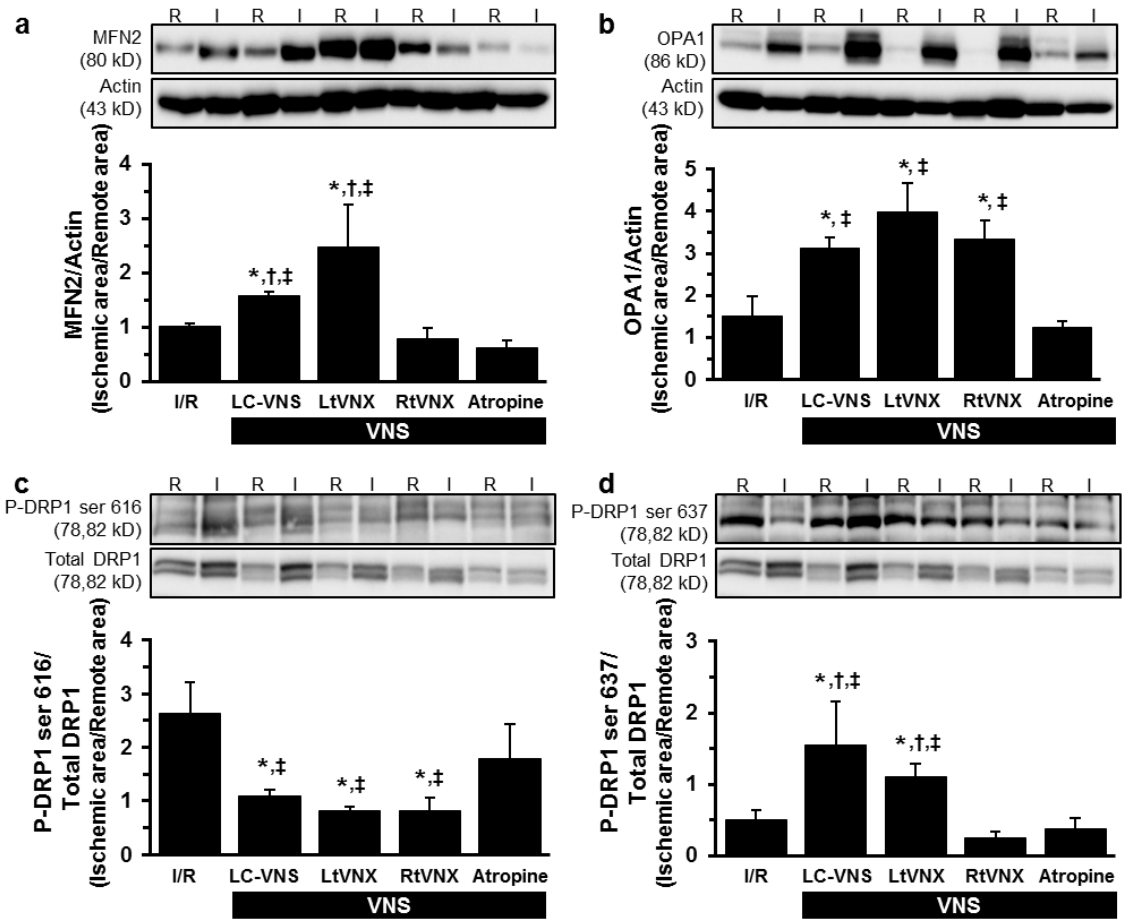

Figure 3-9: Effect of VNS on mitochondrial dynamics. a-d: Representative specific bands and western blot analysis of the mitochondrial dynamics protein expression of OPA1, MFN2, P-DRP1 at Ser 616 and Ser 637. Data are presented as mean ± SE. **P* < 0.05 vs I/R group; †*P* < 0.05 vs RtVNX group; ‡*P* < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

3.10 The effect of VNS on cardiac mitochondrial biogenesis and fatty acid oxidation

The biogenesis of the cardiac mitochondria and fatty acid oxidation were studied by determining the key markers for cellular energy metabolism and fatty acid oxidation (PGC1α and CPT1) (Fig. 10). Both LC-VNS and LtVNX significantly increased the expression of PGC1α compared with the I/R group. However, there was no statistically significant difference in PGC1α level in the RtVNX group. These effects were abolished by atropine.

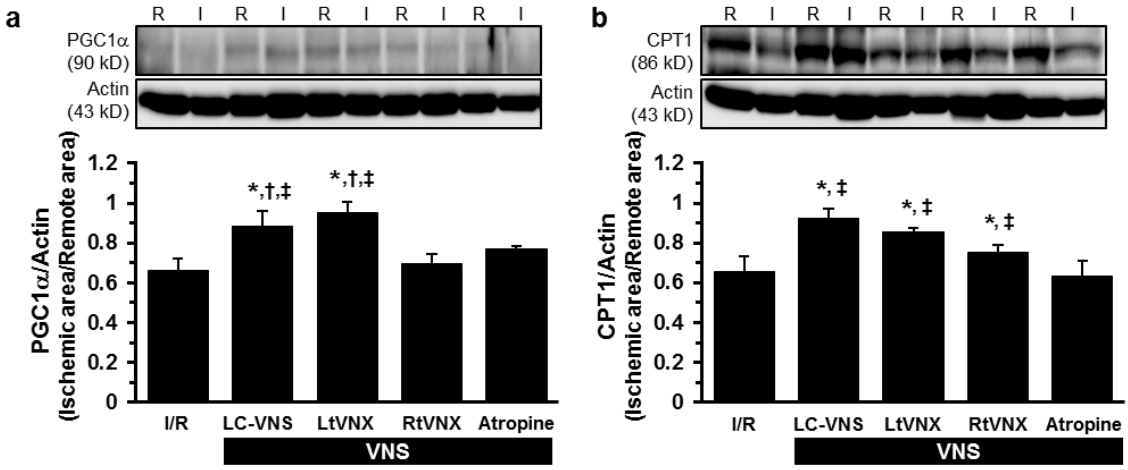

Figure 10: Effects of VNS on myocardium energy metabolism. a and b: Representative immunoblots (top) and densitometric analysis (bottom) of key proteins involved in cardiac mitochondrial beta oxidation (PGC1α and CPT1). Data are presented as mean±SE. *$P < 0.05$ vs I/R group; †$P < 0.05$ vs RtVNX group; ‡$P < 0.05$ vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.