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Clonidine Restores Vascular Endothelial Growth Factor Expression and Improves Tissue Repair following Severe Trauma

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Abstract

Background—We hypothesized that clonidine and propranolol would increase VEGF and VEGF-receptor expression and promote lung healing following severe trauma and chronic stress.

Methods—Sprague-Dawley rats were subjected to lung contusion (LC), lung contusion/hemorrhagic shock (LCHS), or lung contusion/hemorrhagic shock/daily restraint stress (LCHS/CS). Clonidine and propranolol were administered daily. On day seven, lung VEGF, VEGFR-1, VEGFR-2, and HMGB1 were assessed by PCR. Lung injury was assessed by light microscopy (* $p < 0.05$).

Results—Clonidine increased VEGF expression following LCHS (43%*) and LCHS/CS (46%*). Clonidine increased VEGFR-1 and R-2 expression following LCHS/CS (203%* and 47%*, respectively). Clonidine decreased HMGB1 and TNF-alpha expression following LCHS/CS (22%* and 58%*, respectively.) Clonidine decreased inflammatory cell infiltration and total Lung Injury Score following LCHS/CS. Propranolol minimally affected VEGF and did not improve lung healing.

Conclusions—Clonidine increased VEGF and VEGF-receptor expression, decreased HMGB1 expression, decreased lung inflammation, and improved lung tissue repair.

Keywords

Vascular endothelial growth factor; trauma; stress; lung contusion; tissue repair

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Introduction

Vascular endothelial growth factor (VEGF) plays an important role in restoring homeostasis following lung injury by propagating angiogenesis, potentiating pulmonary endothelial cell growth and survival, and stimulating type II alveolar cell surfactant production.^{1, 2} Animal studies have shown that following traumatic lung injury, VEGF and VEGF receptor expression increase over the course of seven days, and there is no histologic evidence of lung injury one week after injury.³ However, when lung contusion (LC) is followed by hemorrhagic shock (HS), VEGF and VEGF receptor expression are decreased, and lung tissue does not heal.³ The addition of chronic stress (CS) to LC and HS further suppresses VEGFR-1, and is associated with greater lung injury. These findings in animal studies are similar to data from human Acute Respiratory Distress Syndrome (ARDS) patients, for whom increased VEGF levels have been associated with resolution of lung injury.⁴ Therefore, restoration of normal VEGF and VEGF receptor expression may be therapeutic for severely injured blunt trauma patients who are subjected to uncontrolled hemorrhage and the daily stressors of the intensive care unit environment.

The purpose of this study was to investigate the effects of two medications that modulate the neuroendocrine stress response, clonidine and propranolol, on VEGF and VEGF receptor expression and lung tissue repair following LC, HS and CS. Propranolol blocks the MAPK, JNK, and NF- κ B stress response pathways occurring downstream of the NE-beta adrenergic receptor complex, and improves burn wound healing.^{5–8} Clonidine is also known to attenuate the inflammatory response following surgery and experimental sepsis, though it has not been studied extensively in wound healing and tissue repair.^{9, 10} Differences in mechanism of action between these medications may also elucidate the relative importance of central sympathetic tone and peripheral adrenergic receptors in stress-mediated wound healing dysfunction. Clonidine reduces central sympathetic tone by stimulating alpha-2 adrenergic receptors in the medulla oblongata and reduces NE release, whereas propranolol has the ability to competitively block the actions of epinephrine and norepinephrine at peripheral beta-adrenergic receptors.^{11, 12} We hypothesized that daily clonidine or propranolol administration in a rodent model of lung contusion/hemorrhagic shock with chronic restraint stress would increase VEGF and VEGF receptor expression, and that these effects would be associated with lung tissue healing.

Methods

Eight week-old male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 300–400g were housed in pairs and fed *ad lib* with Teklad Diet #7912 (Harlan Laboratories Inc., Tampa, FL) and water for a one week acclimation period. Dark and light cycles were 12 hours each during acclimation and experimental periods. All animal care was conducted in accordance with the Institutional Animal Care and Use Committee standards. Animals were randomly allocated ten different groups (n = 6–8 per group): 1) naïve control, 2) lung contusion (LC), 3) LC with clonidine, 4) LC with propranolol, 5) lung contusion followed by hemorrhagic shock (LCHS), 6) LCHS with clonidine, 7) LCHS with propranolol, 8) lung contusion followed by hemorrhagic shock and daily restraint stress (LCHS/CS), 9)

LCHS/CS with clonidine, 10) LCHS/CS with propranolol. Prior to the initial injury, animals were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg). LC was performed by applying a percussive staple gun (PowerShot Model 5700M, Saddle Brook, NJ) to a 12 mm copper plate applied to the right lateral chest wall 1 cm below the axillary crease. This model has previously been shown to produce a clinically significant and reproducible pulmonary contusion.^{13–15}

Rats allocated to HS groups were then placed on a heating pad, and the right internal jugular vein and right femoral artery were cannulated under direct visualization. Continuous blood pressure monitoring was performed by securing the arterial catheter to a BP-2 Digital Blood Pressure Monitor (Columbus Instruments, Columbus, OH). Blood was then withdrawn through the venous catheter into a heparinized syringe until a mean arterial pressure of 30–35 mm Hg was obtained. This blood pressure was maintained for a 45-minute period by withdrawing or reinfusing blood as necessary. After 45 minutes of hemorrhagic shock, blood was reinfused at 1 mL/min. Animals did not receive intravenous or subcutaneous fluids at any point.

CS was performed by placing animals in a restraint cylinder (Kent Scientific Corporation, Torrington, CT) for two hours daily. CS began one day after LCHS in the LCHS/CS group. In order to prevent acclimation to the restraint cylinder, the cylinders were rotated 180 degrees every 30 minutes, and alarms and sirens (80 dB) were transmitted by speakers placed immediately adjacent to the cylinders for two minutes each time the cylinders were rotated. All non-CS groups were subjected to a two hour daily fast while CS was administered.

Clonidine and propranolol were administered by intraperitoneal injection 10 minutes following resuscitation from hemorrhagic shock, and then daily following CS or daily handling. Clonidine and propranolol doses were 75 µg/kg and 10 mg/kg, respectively, based on previous work demonstrating the safety and efficacy of these doses in reducing heart rate by 10–20% without causing significant hypotension.^{13, 16} Propranolol and clonidine were administered once daily rather than more frequent dosing because the goal was to attenuate the neuroendocrine stress response following injury and daily restraint stress rather than to maintain a steady state of pharmacotherapy. Because norepinephrine has a short half-life, a single dose of propranolol or clonidine following resuscitation from hemorrhagic shock or cessation of restraint stress was given.

Animals were sacrificed by cardiac puncture following IP injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) on day seven. Right lung and plasma specimens were collected. Lung specimens were initially placed in phosphate buffered saline (PBS). One portion of the contused right lung was placed in formalin for hematoxylin and eosin staining and histologic analysis by light microscopy, and another portion was placed immediately in dry ice and then stored at –80°C. Plasma samples were obtained during cardiac puncture by withdrawing 7–10 mL of blood into a heparinized syringe. Blood was then centrifuged at 500G for 5 minutes and plasma was aliquoted, placed immediately in dry ice, and then stored at –80°C.

Lung VEGF, VEGFR-1, VEGFR-2, high mobility group box 1 (HMGB1), and tumor necrosis factor alpha (TNF-alpha) expression were assessed by endpoint polymerase chain reaction. HMGB1 expression was measured as a molecular indicator of lung inflammation to correspond with histological findings. The following primers were selected: VEGF forward 5' gtggacttgagtgaggagga and reverse 5' caaacagacttcggcctctc (product region: 2135–2228, product size: 147 bp), VEGFR-1 forward 5' agtggctccacgacctaga and reverse 5' gaagaccgcttcagtttctgt (product region: 2258–2575, product size: 317 bp), VEGFR-2: forward 5' acagcatcaccagcagtcag and reverse 5' ccaagaactccatgccctta (product region: 3127–3274, product size: 147 bp), HMGB1 forward 5' gttctgagtaccgcccaaaa and reverse 5' ttcattctctctgctgtctt (product region: 374–639, product size: 264 bp), TNF-alpha forward 5' gaaacacacgagacgctgaa and reverse 5' ccagatggggatagctgga (product region: 1034–1485, product size: 452 bp). Amplifications were performed using a SimpliAmp thermal cycler (Applied Biosystems, Carlsbad, CA) with an initial 4 minute denaturation phase at 95°C, followed by 32 cycles with denaturation at 95°C, annealing at 60°C, and extension at 72°C for 45 seconds each. Products were separated on 1.5% agarose gel stained with Ethidium Bromide (Invitrogen, Carlsbad, CA). TNF-alpha and HMGB1 were measured on day seven to quantify the persistent inflammatory state following severe trauma and chronic stress to assess if chronic stress was associated with persistent inflammation and impaired wound healing.

Lung injury was assessed by a board certified pathologist using light microscopy to visualize portions of the contused right lung stained with hematoxylin and eosin. The pathologist was blinded to experimental group allocation. The severity of lung injury was analyzed according to the LIS scoring system described in Table 1, adapted from Claridge et al.¹⁷ and Matute-Bello et al.¹⁸

Statistical analysis was performed using GraphPad Prism (version 6.05, GraphPad Software, La Jolla, CA) to calculate one-way analysis of variance. Data were reported as mean $\pm$ standard deviation with $\alpha = 0.05$.

Results

The Effects of Clonidine on VEGF and VEGF Receptors

There was no change in lung VEGF expression following LC only or LC followed by clonidine administration (Figure 1). Seven days following LCHS and LCHS/CS, lung VEGF expression was decreased (Figure 1). With clonidine administration, lung VEGF expression was restored to naïve levels (LCHS + clonidine: 43% increase, $p = 0.003$; LCHS/CS + clonidine: 46% increase, $p = 0.006$; Figure 1).

Lung VEGFR-1 expression did not change seven days after LC when compared to naïve animals (Figure 2A). Lung VEGFR-1 expression was decreased following LCHS and LCHS/CS when compared to naïve animals (Figure 2A). Administration of clonidine increased VEGFR-1 expression following LC, LCHS, and LCHS/CS, though the difference was not statistically significant in the LCHS group (LC + clonidine: 61% increase, $p = 0.01$; LCHS + clonidine: 28% increase, $p = 0.131$; LCHS/CS + clonidine: 203% increase, $p = 0.001$).

Lung VEGFR-2 expression did not change following LC or LC followed by clonidine administration. Lung VEGFR-2 expression was suppressed following LCHS and LCHS/CS, and was significantly increased among LCHS/CS animals that received clonidine, but slightly decreased among LCHS animals that received clonidine when compared to untreated counterpart (LCHS + clonidine: 4% decrease, $p = 0.600$; LCHS/CS + clonidine: 47% increase, $p = 0.004$; Figure 2B).

The Impact of Clonidine on Lung Injury Scores

Following LC alone, total LIS is 0.8 indicating that the injured lung has healed on day seven. Clonidine administration significantly decreased inflammatory cell infiltration (2.4^* vs. 3.8 , $p = 0.011$) and total LIS (5.4^* vs. 8.2 , $p = 0.020$) in LCHS/CS animals when compared to LCHS alone (Table 2). Clonidine administration following LCHS and LCHS/CS was not associated with significant differences in interstitial edema, pulmonary edema, or alveolar integrity (Table 2). Figure 3 includes representative histologic samples comparing LCHS/CS (Figure 3A) to LCHS/CS followed by clonidine administration (Figure 3B) illustrating differences in the number of inflammatory cells and overall lung injury severity.

The Effects of Clonidine on Lung HMGB-1 and TNF-alpha

After making the observation that clonidine decreased lung inflammation, we measured lung HMGB1 and TNF-alpha, which are associated with prolonged inflammation and progression of acute lung injury.^{19, 20} Following acute injury, LC alone and LCHS alone, there was no change in lung HMGB1 expression when compared to naïve (Figure 4). Following injury and chronic stress, lung HMGB1 expression was significantly increased in LCHS/CS group (42% increase, $p = 0.010$; Figure 4). The administration of clonidine following LCHS/CS significantly decreased lung HMGB1 expression when compared to LCHS/CS (22% decrease, $p = 0.031$; Figure 4).

Following LC alone and LC + clonidine there was no change in lung TNF-alpha expression (Figure 5). In the groups with moderate LIS scores on day seven, lung TNF-alpha expression was significantly increased following LCHS (114% increase, $p < 0.001$; Figure 5) and LCHS/CS (107% increase, $p = 0.003$). The administration of clonidine following LCHS and LCHS/CS significantly decreased lung TNF-alpha expression in both groups (LCHS + clonidine: 51% decrease, $p = 0.016$; LCHS/CS + clonidine: 62% decrease, $p = 0.031$; Figure 5).

Propranolol

Administration of propranolol following LCHS did significantly increased lung VEGF expression (33% increase, $p = 0.006$), but not LCHS/CS (25% increase, $p = 0.090$) (Figure 6). The administration of propranolol did increase VEGFR-1 expression following LCHS (31% increase, $p = 0.034$), and LCHS/CS (102% increase, $p = 0.033$), though VEGFR-1 expression remained significantly lower than naïve levels (Figure 7A). Propranolol increased VEGFR-2 following LC, LCHS, and LCHS/CS, but the difference was only statistically significant following LCHS/CS (47% increase, $p = 0.004$) (Figure 7B). Propranolol did not significantly affect any of the individual LIS components or the total LIS (LCHS +

propranolol: 7.6 ± 1.5 , $p = 0.257$ vs. naive; LCHS/CS + propranolol: 9.0 ± 0.0 , $p = 0.189$ vs. naive).

Discussion

Following lung contusion alone, injured lungs have healed by day seven and as expected this study did not demonstrate significant changes in lung VEGF or VEGF receptor expression. Our data did indicate that both clonidine and propranolol impact lung VEGF and VEGF receptor expression following lung contusion/hemorrhagic shock and chronic restraint stress. Daily administration of clonidine more consistently and more significantly increased VEGF and VEGF receptor expression, and also decreased HMGB-1 and TNF- α expression, decreased lung inflammation, and improved lung tissue repair. Lung TNF- α expression on day seven correlated poorly with histologic evidence of lung injury, suggesting that other local factors may play more important roles in restoring homeostasis following lung contusion. Differences between the efficacy of clonidine and propranolol in affecting VEGF and VEGF-receptor physiology and lung tissue repair may be related to differences in their mechanisms of action. Although both medications act on stress response pathways, clonidine acts centrally by blocking sympathetic outflow whereas propranolol functions peripherally at the level of the beta-adrenergic receptor.^{5, 7, 9, 10, 12} Therefore, the superior performance of clonidine is consistent with the hypothesis that the adverse effects of stress-mediated inflammation on wound healing and tissue repair are not limited to beta-adrenergic receptor modulation and downstream pathways like MAPK, JNK, and NF- κ B.

In previous studies, persistently low levels of alveolar neutrophil VEGF (6.3 pg/mL) have been associated with prolonged illness in human subjects with Acute Respiratory Distress Syndrome (ARDS), whereas increased levels (13.0 pg/mL) correlated with recovery four days after onset of symptoms.⁴ Conversely, overexpression of lung VEGF has been associated with pulmonary edema in an animal model utilizing intratracheal delivery of VEGF complementary protein by an adenovirus vector.²¹ Therefore, too much or too little VEGF may be detrimental to tissue repair. In our model, the addition of HS and CS to LC appear to be associated with a pathologic decrease in VEGF and VEGF receptor expression, and clonidine restores expression levels and improves lung healing.

Previous work has shown that lung VEGF and VEGF receptors may be modulated by potentiating nitric oxide or by inhibiting nitric oxide synthase in the settings of acute and chronic hypoxia.²² In addition, exogenous administration of a pannexin-1 blocker, carbenoxolone, has been shown to decrease LPS-induced HMGB1 release and attenuate LPS-induced acute lung injury.^{23, 24} However, modulation of VEGF, VEGF receptors, and HMGB1 to promote tissue repair following traumatic lung injury have not been previously reported. The authors surmise that clonidine did not improve lung tissue repair following LCHS because VEGF receptor 1 and 2 expression were not significantly increased by the addition of clonidine following LCHS. This observation supports the hypothesis that both VEGF and VEGF receptor expression play important roles in lung tissue repair following severe trauma.

The major limitations of this study are the lack of mechanistic detail and causal relationships. Also, it may be difficult to translate these findings to the clinical setting, as clonidine may propagate hypotension and has effects lasting for 6–10 hours, such that patient selection may be challenging. Although previous work suggests that direct tissue injury is responsible for changes in VEGF and VEGF receptor expression, closer investigation of stress response pathways upstream of the beta-adrenergic receptor may clarify the mechanism by which clonidine increased VEGF and VEGF receptor expression and improved lung tissue repair following LCHS/CS. Previous work suggests that clonidine may inhibit mast cell degranulation and neutrophil chemotaxis.²⁵ Also, hemorrhagic shock was not performed as an isolated physiologic insult, though it would be interesting to assess the effects of hemorrhagic shock alone on VEGF and VEGF receptor expression following hemorrhagic shock alone. This scenario may apply to trauma patients with major vascular injuries due to penetrating trauma. In addition, these experiments were performed using whole lung samples rather than bronchoalveolar lavage fluid specimens, and assessment of lavage fluid would contribute to our understanding of the pathophysiology described in this study. Finally, hematopoietic progenitor cell mobilization and T regulatory cell function appear to play a role in lung tissue repair,^{14, 15, 26} and the addition of mesenchymal stem cell administration could provide useful insights.

Conclusions

Clonidine increased lung VEGF and VEGF receptor expression following severe injury and chronic stress, decreased inflammatory cell infiltration, and improved tissue repair. Propranolol was less effective in restoring VEGF and VEGF receptor expression levels and did not improve lung tissue repair, suggesting that the stress response pathways responsible for wound healing dysfunction following severe injury may occur upstream of the beta-adrenergic receptor. Future studies should seek to establish causality of VEGF physiology in lung tissue healing and clarify the mechanism by which clonidine increases VEGF and VEGF receptor expression.

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Summary

We hypothesized that clonidine and propranolol would increase VEGF and VEGF-receptor expression and promote lung healing in Sprague-Dawley rats subjected severe trauma and chronic stress. Clonidine increased VEGF and VEGF-receptor expression, decreased HMGB1 expression, decreased lung inflammation, and improved lung tissue repair. Propranolol did not significantly affect VEGF or lung healing.

Figure 1.

Clonidine increased lung VEGF expression seven days following LCHS and LCHS/CS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Figure 2.

Figure 2A. Clonidine increased lung VEGFR-1 expression seven days following LC and LCHS/CS. **2B:** Clonidine increased lung VEGFR-2 expression seven days following LCHS/CS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Figure 3. Clonidine significantly decreased inflammatory cell infiltration and improved overall lung tissue repair seven days following lung contusion, hemorrhagic shock, and chronic stress (**A**: without clonidine, **B**: with clonidine).

Figure 4.

Clonidine decreased HMGB1 expression following LCHS/CS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Figure 5.

Clonidine decreased TNF-alpha expression following LCHS and LCHS/CS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Figure 6.

Propranolol increased lung VEGF expression seven days following LCHS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Figure 7.

Figure 7A. Propranolol increased lung VEGFR-1 expression seven days following LCHS and LCHS/CS. **7B:** Clonidine increased lung VEGFR-2 expression seven days following LCHS/CS (LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, Clon: clonidine, * $p < 0.05$ vs. naive, ** $p < 0.05$ within the group).

Table 1

Lung Injury Score calculator adapted from Claridge et al.¹⁷ and Matute-Bello et al.¹⁸ HPF: high-power field.

Components	Points
Inflammatory cells/HPF	
<5	0
6 to 10	1
11 to 15	2
16 to 20	3
>20	4
Interstitial edema	
None	0
Minimal	1
Moderate	2
Severe	3
Pulmonary edema	
<5%	0
5–25%	1
>25%	2
Alveolar integrity	
Normal	0
Moderately abnormal	1
Severely abnormal	2

Table 2

Clonidine improved lung tissue repair following LCHS/CS. LC: lung contusion, HS: hemorrhagic shock, CS: chronic stress, ^a $p = 0.011$ vs. LCHS/CS, ^b $p = 0.020$ vs. LCHS/CS. Data are reported as mean $\pm$ standard deviation.

Components	LCHS	LCHS + Clonidine	LCHS/CS	LCHS/CS + Clonidine
Inflammatory cells/HPF (naïve: 0.3 ± 0.5)	3.3 ± 0.5	3.0 ± 1.3	3.8 ± 0.4	2.4 ± 0.9^a
Interstitial edema (naïve: 0.0 ± 0.0)	2.0 ± 0.0	2.0 ± 0.8	2.2 ± 0.4	1.6 ± 0.5
Pulmonary edema (naïve: 0.7 ± 0.5)	0.0 ± 0.0	0.7 ± 0.8	0.3 ± 0.5	0.2 ± 0.4
Alveolar integrity (naïve: 0.0 ± 0.0)	1.3 ± 1.0	1.6 ± 0.8	1.8 ± 0.4	0.8 ± 0.4
Total Lung Injury Score (naïve: 1.0 ± 0.9)	6.6 ± 1.0	7.3 ± 2.1	8.2 ± 0.8	5.4 ± 2.2^b