ELUCIDATION OF MECHANISM UNDERLYING
PEROXYNITRITE MEDIATED IMPAIRED RELAXATION IN
THORACIC AORTA OF ARTHRITIC RATS


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ABSTRACT
Rheumatoid arthritis is a systemic inflammatory disease associated with generation of oxidative stress that produced vascular dysfunction. ONOO· a cytotoxic free radical and potent vasorelaxant. The present study was undertaken to determine the mechanism underlying ONOO· induced vascular relaxation in rat aorta with probable mechanism involved in impaired relaxation in arthritic rat aorta. Arthritis was induced by CFA (6 mg/ml, i.d.). After precontraction of aortic strips by PE (10-5 M), vasodilatory response to peroxynitrite was taken. The strip of aorta was incubated with various potassium channel blockers such as TEA (Ca2+ activated potassium channel blocker), Glibenclamide (selective ATP dependent potassium channel blocker), 4-AP (Voltage dependent potassium channel blocker), BaCl2 (Inwardly rectifying Potassium channel blocker) and Methylene blue (sGC blocker). ONOO· induced relaxation was impaired in arthritic compared to control rat aorta, suggested that vascular dysfunction in Arthritis. In presence of Methylene blue, significantly inhibited ONOO· induced relaxation in control and arthritic thoracic aorta, suggested the role of sGC in ONOO· induced relaxation. TEA (10mM) and 4-AP (10-5 M) significantly inhibited ONOO· induced relaxation in both arthritic and control rat aorta. While in presence of glibenclamide (10-5 M) and BaCl2 (0.1mM) ONOO· relaxation was unaltered indicates that ONOO· induced relaxation may be via KCa and KV channel but independent of KATP and KIr channels. ONOO· induced relaxation involves activation of sGC and potassium channel particularly through KCa and KV channel. In arthritic rat aorta, impairment of ONOO· induced relaxation due to dysfunction in KCa and KV channels.

Key words: Rheumatoid arthritis, Vascular Dysfunction, Peroxynitrite, Potassium Channel.
INTRODUCTION

Rheumatoid arthritis is a systemic inflammatory disease characterized by chronic and erosive synovitis that involves peripheral joints\cite{1}. The incidence of RA is 30 cases per 10,000 populations. The disease is 3 times more common in women than men. Inflammations play a key role in the pathogenesis of RA. Recent studies have suggested that vascular inflammation in RA patients often lead to vascular endothelial injuries and vascular dysfunction. Arthritis is closely associated with increased oxidative and nitrosative stress, which can trigger the development of endothelial injury and vascular dysfunction\cite{2}. RA is associated with an increased mortality from cardiovascular causes\cite{3,4}. Recent reports demonstrate that endothelial function is reduced in RA patients with high inflammatory activity\cite{5}. Although the underlying mechanisms for endothelial dysfunction in RA are poorly understood, it is postulated that systemic inflammation may be involved in the early vascular damage\cite{6}. Endothelium plays a pivotal role in the regulation of vascular tone and structure through the release of various vasoactive agents, such as vasodilators and vasoconstrictors. It has been recognized that alterations of endothelial function are involved in the development and progression of atherosclerosis and its clinical complications\cite{7}. Endothelial dysfunction is considered to represent reduced bioavailability of nitric oxide (NO), which is a major endothelium-dependent vasodilator. Endothelium-dependent NO is also known to have other anti atherosclerotic properties, including inhibition of cell growth, leukocyte adhesion, and platelet adherence and aggregation\cite{8}. It has been reported that vascular production of ROS, such as O$_2^-$, is increased in hypertension, atherosclerosis, diabetes and arthritis\cite{9}. O$_2^-$ reacts rapidly with NO, resulting in the formation of ONOO\(^-\), which could lead to a loss of bioactivity of NO. The increased oxidative stress may affect the synthesis of NO. Vascular NO is mainly synthesized by eNOS from the precursor L-arginine. However, previous data demonstrate that increased oxidative stress promotes a dysfunctional eNOS, which generates O$_2^-$ instead of NO\cite{10}. This dysfunctional enzyme is termed uncoupled eNOS. It is reported that the formation of uncoupled eNOS is linked to oxidative degradation of the critical eNOS cofactor tetrahydrobiopterin\cite{11}.

Animal models have been essential to understanding the pathophysiologic mechanisms of human diseases. To elucidate the mechanism of vascular dysfunction
caused by systemic inflammation, we used AIA in the rat, which has been widely used as a model of RA. The experiments conducted in the study were designed to determine endothelium independent relaxation is impaired in the vasculature of the arthritis model, To determine whether vascular oxidative stress is increased in AIA, To identify which type of Potassium channel is disturb in RA and the role of sGC in vascular dysfunction.

MATERIAL AND METHOD

Animals

Female Sprague Dawley rats of weighing 250-300g were procured from central animal facility of S. K. Patel College of Pharmaceutical Education and Research. Experimental protocols were approved by IAEC.

Chemicals

Freund’s adjuvant (Difco Laboratories Detroit, Michigan, U.S.A), Methylene blue (Soluble Guanyl cyclase inhibitor), Tetraethyl ammonium (Ca$^{2+}$ activated potassium channel blocker), Glibenclamide (selective ATP dependent potassium channel blocker), 4-AP (Voltage dependent potassium channel blocker), BaCl$_2$ (Inwardly rectifying Potassium channel blocker) were procured from Sigma-Aldrich, U.S.A. ONOO$^-$ was synthesized in the laboratory as method describe by Beckman et.al, 1994.$^{[12]}$

Experimental Design

Induction of Adjuvant Arthritis in Rats.$^{[13]}$

Female Sprague Dawley rats were used. On 1$^{st}$ day, they were injected into the sub plantar region of the left hind paw with 0.1 ml of CFA (6 mg/ml). On 28th day, the ESR, Body Weight, Paw Volume, RA Factor and Arthritic Index were measured.

Measurement of various parameters

Body weight and Inflammation were measured.$^{[14]}$ Blood pressure was measured by non-invasive tail-cuff method, Arthritic Index was observed.$^{[15]}$ and ESR was measured by westergren method. RA Factor, Serum Homocysteine Level and Hematological parameter were measured in pathology laboratory.

Anti-oxidant activity

Superoxidedismutase was measured.$^{[16]}$, Catalase was measured.$^{[17]}$. 
Vascular reactivity study

After 28\textsuperscript{th} day of CFA induction, rats were sacrificed by cervical dislocation and thoracic aorta was isolated and prepared a spiral strip of 2.5 cm segments. Strip was mounted in the organ bath and equilibrates for 2 hours, after equilibration two-equipotent responses of KCl (80mM) were taken to check the stability of the tissue. After 15 min of interval concentration response curve for relaxation by ONOO\textsuperscript{−} on aorta precontracted with PE (10\textsuperscript{−}5M) \cite{18} were constructed in the control animal with intact and denuded endothelium aorta and arthritic aorta. Drugs such as Methylene blue, Glibenclamide, TEA, BaCl\textsubscript{2} and 4-AP were preincubated for 20 minutes, before concentration response curve of ONOO\textsuperscript{−} were taken in PE precontracted strip, studies were carried out in arthritic aorta. ONOO\textsuperscript{−} induced relaxation were only taken when PE induced contraction remain persistent after reaching to maximum.

Analysis of dose response curves

The measured relaxation in mm was expressed in Mean ± S.E.M for calculation of % Relaxation and Rmax in both control and arthritic rats. The % Relaxation was calculated with the maximal relaxation to ONOO\textsuperscript{−} as 100% in both control and in arthritic rats. % Relaxation for each experiment was plotted against log [M] conc. of the ONOO\textsuperscript{−}. This was useful for the calculation of pD\textsubscript{2} value. % Inhibition of relaxation was calculated for control and arthritic rats in the presence of specific blockers and compared.

Statistical analysis

The results were expressed as mean ± S.E.M. Statistical difference between two means determined by unpaired t-test using software Graph pad prism. p<0.05 was considered as statistically significant.
RESULTS
Comparison of different parameter after 28\textsuperscript{th} day.

Table 1: Data showing comparison of bodyweight, Paw Volume, RA Factor, ESR, Serum Homocysteine, Haematological parameter (Hb, RBC, WBC) and Systolic blood pressure in control and arthritic rats.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameter</th>
<th>Control</th>
<th>Arthritic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Body weight (gm)</td>
<td>273.3 ± 1.173</td>
<td>229.1 ± 1.068***</td>
</tr>
<tr>
<td>2</td>
<td>Paw Volume (ml)</td>
<td>0 Day 0.8767 ± 0.0024</td>
<td>0.8767 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>28\textsuperscript{th} Day</td>
<td>0.8013 ± 0.0196</td>
<td>2.140 ± 0.0688***</td>
</tr>
<tr>
<td>3</td>
<td>RA Factor (IU/ml)</td>
<td>2.485 ± 0.1300</td>
<td>13.93 ± 0.9724***</td>
</tr>
<tr>
<td>4</td>
<td>ESR (1hr)</td>
<td>2.500 ± 0.1946</td>
<td>9.417 ± 0.4680***</td>
</tr>
<tr>
<td>5</td>
<td>Serum Homocysteine (µM/L)</td>
<td>8.020 ± 0.5600</td>
<td>12.74 ± 0.6200***</td>
</tr>
<tr>
<td>6</td>
<td>Haematological Parameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hb (gm%)</td>
<td>16.18 ± 0.1523</td>
<td>12.72 ± 0.4623***</td>
</tr>
<tr>
<td></td>
<td>RBC (Million/cmm)</td>
<td>5.375 ± 0.1338</td>
<td>5.808 ± 0.2032</td>
</tr>
<tr>
<td></td>
<td>WBC (Thousand/cmm)</td>
<td>7.880 ± 0.1595</td>
<td>11.53 ± 0.4109***</td>
</tr>
<tr>
<td>7</td>
<td>Systolic Blood pressure (mmHg)</td>
<td>117.1 ± 0.8467</td>
<td>147.6 ± 0.5548***</td>
</tr>
<tr>
<td>8</td>
<td>SOD</td>
<td>6.58 ± 0.1338</td>
<td>3.110 ± 0.2560 ***</td>
</tr>
<tr>
<td>9</td>
<td>Catalase</td>
<td>6.92 ± 0.4680</td>
<td>3.650 ± 0.198***</td>
</tr>
</tbody>
</table>

Values are expressed in Mean ± S.E.M. N = 6-12. Arthritic animals show significant difference in bodyweight, Paw Volume, RA Factor, ESR, Serum Homocysteine, Haematological parameter (Hb, RBC, WBC) and Systolic blood pressure as compared to control. ***P<0.001 Vs. control.

After 28\textsuperscript{th} Day of single dose administration of CFA (6mg/ml, i.d.). The symptoms of arthritis like loss of body weight and severe inflammation was found.

VASCULAR REACTIVITY STUDY
Peroxynitrite induced relaxation on aorta precontracted with KCl (80mM)

![Figure 1](image_url)  

Concentration response curve of ONOO\textsuperscript{-} (10\textsuperscript{-9}M to 10\textsuperscript{-3}M) induced relaxation in control rat aortic strips, precontracted with KCl (80mM).
Comparison of peroxynitrite induced relaxation in presence and absence of endothelium

**Figure 2**
Concentration response curve of ONOO\(^-\) (10\(^{-9}\)M to 10\(^{-3}\)M) induced relaxation in control rat aortic strips, precontracted with PE (10\(^{-5}\) M) in endothelium intact and denude preparation.

Comparison of peroxynitrite induced relaxation in arthritic and control aorta

**Figure 3**
Concentration response curve of ONOO\(^-\) (10\(^{-9}\)M to 10\(^{-3}\)M) induced relaxation in control and arthritic aortic strips, precontracted with PE (10\(^{-5}\) M).
Effect of TEA on peroxynitrite induced relaxation in control and arthritic aorta

Figure 4(a)
Concentration response curve of ONOO\(^-\) (10\(^{-9}\)M to 10\(^{-3}\)M) induced relaxation in control and control incubated with TEA (10mM) aortic strips, precontracted with PE (10\(^{-5}\) M).

Figure 4(b)
Concentration response curve of ONOO\(^-\) (10\(^{-9}\)M to 10\(^{-3}\)M) induced relaxation in arthritic and arthritic incubated with TEA (10mM) aortic strips, precontracted with PE (10\(^{-5}\) M).

Effect of Glibenclamide on peroxynitrite induced relaxation in control and arthritic aorta

Figure 5(a)
Concentration response curve of ONOO\(^-\) (10\(^{-9}\)M to 10\(^{-3}\)M) induced relaxation in control and control incubated with glibenclamide (10-5 M) aortic strips, precontracted with PE (10\(^{-5}\) M).
Concentration response curve of ONOO$^-$ (10$^{-9}$M to 10$^{-3}$M) induced in arthritic and arthritic incubated with glibenclamide (10$^{-5}$ M) aortic strips, precontracted with PE (10$^{-5}$ M).

Effect of BaCl$_2$ on peroxynitrite induced relaxation in control and arthritic aorta

Concentration response curve of ONOO$^-$ (10$^{-9}$M to 10$^{-3}$M) induced relaxation in control and control incubated with barium chloride (0.1mM) aortic strips, precontracted with PE (10$^{-5}$ M).

Concentration response curve of ONOO$^-$ (10$^{-9}$M to 10$^{-3}$M) induced relaxation in arthritic and arthritic incubated with barium chloride (0.1mM) aortic strips, precontracted with PE (10$^{-5}$ M).
Effect of 4-AP on peroxynitrite induced relaxation on control and arthritic aorta

**Figure 7(a)**
Concentration response curve of $\text{ONOO}^{-}$ ($10^{-9}$M to $10^{-3}$M) induced relaxation in control and control incubated with 4-aminopyridine ($10^{-5}$ M) aortic strips, precontracted with PE ($10^{-5}$ M).

**Figure 7(b)**
Concentration response curve of $\text{ONOO}^{-}$ ($10^{-9}$M to $10^{-3}$M) induced relaxation in arthritic and arthritic incubated with 4-aminopyridine ($10^{-5}$ M) aortic strips, precontracted with PE ($10^{-5}$ M).

Effect of Methylene blue on peroxynitrite induced relaxation on control and arthritic aorta

**Figure 8(a)**
Concentration response curve of $\text{ONOO}^{-}$ ($10^{-9}$M to $10^{-3}$M) induced relaxation in control and control incubated with Methylene blue (10μM) aortic strips, precontracted with PE ($10^{-5}$ M).
Concentration response curve of ONOO⁻ (10⁻⁹ M to 10⁻³ M) induced relaxation in arthritic and arthritic incubated with Methylene Blue (10µM) aortic strips, precontracted with PE (10⁻⁵ M).

Comparison of % inhibition of peroxynitrite induced relaxation in presence of its specific blocker

Data represents the % inhibition of relaxation of KCa blocker (TEA), KV blocker (4-AP), Kir channel blocker (BaCl₂), KATP channel blocker (Glibenclamide) and Methylene blue (sGC blocker) on ONOO⁻ induced relaxation in the aorta of arthritic and control.

\* P< 0.05, \** P< 0.01, \*** P< 0.001.

pD₂ values of ONOO⁻ in control and diabetic aortas in presence of its specific blockers

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment</th>
<th>Control pD₂ Values</th>
<th>Arthritic pD₂ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Endothelium intact</td>
<td>5.546</td>
<td>5.704</td>
</tr>
<tr>
<td>2</td>
<td>TEA (10mM)</td>
<td>5.835</td>
<td>5.887</td>
</tr>
<tr>
<td>3</td>
<td>Glibenclamide (10⁻⁵ M)</td>
<td>6.0529</td>
<td>6.366</td>
</tr>
<tr>
<td>4</td>
<td>BaCl₂ (0.1mM)</td>
<td>5.423</td>
<td>5.666</td>
</tr>
<tr>
<td>5</td>
<td>4 – Amino Pyridine (10⁻³M)</td>
<td>5.916</td>
<td>6.324</td>
</tr>
<tr>
<td>6</td>
<td>Methylene blue (10µM)</td>
<td>5.780</td>
<td>6.27</td>
</tr>
<tr>
<td>7</td>
<td>Methylene blue (20µM)</td>
<td>5.558</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Endothelium denuded</td>
<td>5.753</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION

In present study we observed that, after 28th day significant reduction in body weight and increase paw volume, RA Factor, ESR compare to control rat (Table 1) indicates the symptoms of arthritic condition. Vascular dysfunction was shown from the increase Homocysteine level (Table 1). Decreased activity of SOD and catalase in arthritis can leads to excess availability of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in the biological system, which in turns generates the \( \text{OH}^- \) resulting in the propagation of Lipid peroxidation and ultimate the production of oxidative stress.

Jiangfeng Li et al, 2005 had shown that the mechanism behind the vasorelaxation effect of ONOO\(^-\) on rat cerebral arteries\(^{[19]}\). He stated that the ONOO\(^-\) exerted vasodilation due to activation of potassium channel, myosin phosphatase and elevation of sGC.

Vascular dysfunction in AIA is independent of constitutive or inducible prostanoid mechanism and appears not to be solely endothelium derived but to involve other components such as the vascular smooth muscles\(^{[20]}\). Endothelial dysfunction is considered to represent reduction of bioavailability of nitric oxide\(^{[21]}\). So with special emphasis of ONOO\(^-\) induced relaxation in arthritic aorta, study was carried out to elucidate potassium channel dysfunction. In the absence of any vasoactive agent, ONOO\(^-\) failed to alter the basal tension over wide range of concentration in both control and arthritic aorta. As shown in Figure 2 ONOO\(^-\), however resulted in a vasorelaxant effect on PE (10\(^{-5}\)M) precontracted rat aortic strip. The cumulative addition of (10\(^{-9}\)M to 10\(^{-3}\)M) ONOO\(^-\) led to a concentration dependent relaxation, reaching a maximum of 100%. These results were consistent with previous reports obtained in another types of arterial rings from different animals\(^{[22, 23]}\). It is known that endothelium can release NO and \( \text{PGI}_2 \), and that both can lead to smooth muscle relaxation\(^{[24]}\). Mechanical disruption of intimal endothelium did not alter ONOO\(^-\) induced vasorelaxation (Figure 2) and \( R_{\text{max}} \) was identical as observed in endothelium intact rat aortic strip. These results indicating that endothelium have no mediating action on the relaxation action of ONOO\(^-\). This conclusion is in consistent with previously performed study in cerebral arteries in presence of L-NAME, L-arginine and indomethacin which are NOs inhibitor, NO activator and cycloxygenase inhibitor respectively\(^{[19]}\). So, further study was carried out in
the presence of endothelium intact preparation in rat aortic strip. Figure 3, shows that ONOO⁻ induced relaxation was significantly inhibited in arthritic aorta as compared to control. From that we have found that the vascular dysfunction was present in AIA. This finding suggest that impaired ONOO⁻ induced relaxation in arthritic rat aortic strip may be due to impairment in one of the mechanism that is responsible for peroxynitrite induced relaxation in control rat aortic strip. It is well documented that the intracellular accumulation of cGMP signals, protein kinase, ion channels, and other effectors systems to cause smooth muscle relaxation [25, 26, 27, 28]. So, attempt to probe such possible mechanism of ONOO⁻ induced vasorelaxation with respect to cGMP. It has been established that many stimulants activate guanyl cyclase resulting in formation of cGMP and in turn it can mediated smooth muscle relaxation via regulation of Ca⁺² and other factors. The concentration of cGMP is controlled through the rate of synthesis of guanyl cyclase and the rate of hydrolysis to guanosine 5-monophosphate by nucleotide phosphodiesterases [29].

In our experiments, Methylene blue is the inhibitor of sGC, significantly inhibited ONOO⁻ induced relaxation in control (Figure 8(a)) and arthritic thoracic aorta (Figure 8(b)). This suggests that the relaxant effects of ONOO⁻ on rat aortic strip may be via activation of sGC in the rat aortic smooth muscle cells. These results are in consistant with previously reported study on cultured vascular smooth muscle cells [28] and reports on cerebral arteries [19].

In preliminary study, ONOO⁻ induced relaxation was impaired in rat aortic strip precontracted with KCl (80mM) (Figure 1), pointing towards involvement of potassium channel. This finding suggest that peroxynitrite induced relaxation may involve the role of potassium channel. In recent year, several electrophysiological and pharmacological studies have demonstrated an important role of potassium channel in the hyperpolarisation mediated relaxation of vascular smooth muscle [30]. Cerebral arterial dilation through opening of K_ATP channel [31]. Jiangfeng et al., 2005 had also demonstrated role of potassium channel in ONOO⁻ induced relaxation in cerebral arteries and thoracic aorta [19].

ONOO⁻ induced relaxation was inhibited by TEA (Figure 4(a, b)) and 4-AP (Figure 7(a, b)) in both control and arthritic rat. While ONOO⁻ induced relaxation were
not inhibited in the presence of glibenclamide (Figure 5(a, b)) and BaCl$_2$ (Figure 6(a, b))
in both arthritic and control rats. These indicate that ONOO$^-$ induced relaxation is
mediated by activation of $K_{Ca}$, $K_{V}$ and also suggest that relaxation may independent of
$K_{ATP}$ and $K_{ir}$.

Comparison of % inhibition of relaxation in presence of its specific blockers suggest that impairment in relaxation in arthritic aorta is significantly more as compared
to control aorta in presence of TEA , 4-AP, Methylene Blue (Figure 9). This finding
suggests that dysfunction in $K_{Ca}$ and $K_{V}$ channel occur which is responsible for
cardiovascular dysfunction associated with arthritic condition.

CONCLUSION

Due to Prolonged inflammation, rats have been developed oxidative stress leading
to vascular dysfunction. ONOO$^-$ induced relaxation is independent of
endothelium. Peroxynitrite induced relaxation was impaired in arthritic compared to
control aorta that indicates vascular dysfunction. Mechanism underlying peroxynitrite
induced relaxation response were activation of sGC activity proved by Methylene blue
and activation of $K_{V}$ and $K_{Ca}$ proved by TEA and 4-AP while there was no role of $K_{ATP}$
and $K_{ir}$. Impairment in ONOO$^-$ induced relaxation in presence of TEA, 4-AP and
Methylene blue were more in arthritic aorta as compared to control that indicates
dysfunction in $K_{Ca}$, $K_{V}$ channels and role of cGMP in arthritic condition that is
responsible for cardiovascular dysfunction. So, potassium channel opener selectively
acting on vascular smooth muscle cell may be therapeutic approach in cardiovascular
complication associated with arthritis.

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