Inhibitory Effect of Ammonium Chloride on Acetylcholine-Induced Relaxation

Katsuyuki Ando, Toshiro Fujita

Abstract We designed the present study to clarify whether the intracellular pH change by ammonium chloride influences endothelium-dependent relaxation in thoracic aorta of 9-week-old Sprague-Dawley rats. Intracellular alkalinization with 3 mmol/L ammonium chloride, which did not affect resting vascular tone, attenuated acetylcholine-induced relaxation but not nitrroglycerin vasodilation. Acetylcholine relaxation was more inhibited by a shorter duration of treatment. Thus, change in intracellular pH may be important in the effect because the alkalinizing effect of ammonium chloride disappears gradually. In support of this, the proton ionophore nigericin abolished the effect. Also, amiloride shortened the effect of ammonium chloride, suggesting that intracellular pH plays a role: sodium-proton antiport antagonizes the disappearance of ammonium chloride-induced intracellular alkalinization. The synthesis of vasoconstrictor prostaglandins, such as thromboxane A2, may be stimulated during acetylcholine treatment, resulting in the attenuation of acetylcholine relaxation, because the relaxation was abolished by treatment with the phospholipase A2 inhibitor quinacrine, cyclooxygenase inhibitor indomethacin, prostaglandin H2/thromboxane A2 receptor antagonist S1452, and thromboxane A2 synthase inhibitor dazmegrel. Phospholipase A2 may contribute to the effect of intracellular alkalinization, which is compatible with the fact that the optimal pH of phospholipase A2 is neutral to alkaline. In addition, superoxide dismutase attenuated the effect of ammonium chloride. In conclusion, intracellular alkalinization by ammonium chloride attenuated acetylcholine-induced relaxation, possibly through the interrelated production of both thromboxane A2 and superoxide radicals. (Hypertension. 1994;24:189-194.)

Key Words • endothelium • proton concentration • prostaglandins • superoxide • ion channels • rats, Sprague-Dawley

Moreover, some vasoconstrictors, which caused intracellular alkalinization, have been reported to produce prostaglandins.9,10 Also, phospholipase A2 (PLA2), which produces arachidonate and facilitates prostaglandin generation, is activated by intracellular alkalinization, because the optimal pH of this enzyme is neutral to alkaline.11,12 In addition, increased pH, stimulated the generation of superoxide radicals in human neutrophils.13 Thus, vasoconstrictor prostaglandins and/or superoxide may be involved in alkalinization-induced vasoconstriction through endothelial cells. To clarify this hypothesis, we examined the effect of NH4Cl on acetylcholine-induced relaxation and its involvement with prostaglandins and superoxide in rat thoracic aorta.

Methods

Preparation of Aortic Rings

Aortic rings of 9-week-old male Sprague-Dawley rats (Charles River Japan; approximately 280 to 320 g) were prepared for isometric tension recording with a previously reported standard organ bath method.8,14 Briefly, rats were killed with light ether anesthesia, and the thoracic aorta was removed. In Krebs' bicarbonate solution, the aorta was divided into four segments 4 to 5 mm long. The rings were carefully handled to avoid damage to the inner surface. In some rings, endothelium was removed mechanically by rubbing with cotton. The rings were suspended in organ bath chambers containing 8 mL Krebs' bicarbonate buffer of the following composition (mmol/L): NaCl 112, NaHCO3 25.2, KCl 4.73, MgCl2 1.19, KH2PO4 1.19, CaCl2 0.9, EDTA 0.026, and dextrose 11.0. The medium was equilibrated to pH 7.40 by continuous aeration with a mixture of 95% O2/5% CO2 and maintained at 37°C. The upper wire supporting each ring was attached to a force-displacement transducer (TB-651T, Nihon-Kohden), and changes in isometric force were displayed on a chart recorder (WT-685G, Nihon-Kohden). Resting force of aortic rings was 1.0 g; rings were allowed to equilibrate for 90 minutes. Then rings were exposed...
to a depolarizing concentration of KCl (60 mmol/L) and washed with Krebs' solution. After 20 minutes, the aortic rings were contracted by 3×10⁻⁷ mol/L norepinephrine, and, when the contractile response was stabilized, 10⁻⁶ mol/L acetylcholine was applied to the rings for examination of whether functional endothelium was present. For the following experiment, we used rings with intact endothelium, in which a sufficient relaxation (>80% of norepinephrine contraction) was obtained by acetylcholine application, and rings without endothelium, which had no relaxation by acetylcholine. Twenty minutes after the chambers were washed at least three times with Krebs' solution, the experiments were started. Endothelium-derived relaxation was calculated by cumulative addition of acetylcholine (10⁻⁴ to 10⁻² mol/L) in precontracted aortic rings by 3×10⁻⁷ mol/L norepinephrine. In some rings, endothelium-unrelated relaxation was examined by nitroglycerin (10⁻⁹ to 10⁻⁷ mol/L) after norepinephrine precontraction.

Experimental Protocols

**Experiment 1: Effect of Changes in pH on Acetylcholine Relaxation**

First, we examined the effect of the different NH₄Cl doses (3, 5, 10, and 20 mmol/L, 10 minutes) on norepinephrine contraction (3×10⁻⁷ mol/L). Intracellular alkalinization was induced by a small dose (3 mmol/L) of NH₄Cl (10 minutes) because a high dose (>10 mmol/L) affected vascular tone (see "Results") and vascular reactivity (Fig 1) in rat aorta, similar to previous reports. Acidification was induced by the removal of 3 mmol/L NH₄Cl (10 minutes after its administration) and treatment (10 minutes) with 10⁻⁷ mol/L nigericin. After these treatments, cumulative acetylcholine relaxation was examined. Nitroglycerin relaxation was also examined after NH₄Cl treatment, as was acetylcholine relaxation in NH₄Cl-treated aorta without intact endothelium.

**Experiment 2: Time-Dependent Effect of NH₄Cl Treatment on Acetylcholine Relaxation**

Intracellular alkalinization by NH₄Cl gradually disappeared, so we examined the effects of different treatment periods of NH₄Cl (0-minute and 10-minute treatments) on acetylcholine relaxation. Norepinephrine precontraction was started immediately after NH₄Cl administration in the 0-minute treatment and 10 minutes after NH₄Cl administration in the 10-minute treatment. Because accurate treatment time is critical in experiment 2, acetylcholine relaxation began 3 minutes after norepinephrine precontraction, and cumulative acetylcholine application was done each 1 minute. Acetylcholine relaxation was also examined after inhibition of NH₄Cl (0-minute and 10-minute treatments)–induced alkalinization by 10⁻⁴ mol/L nigericin. Moreover, we tested the influence of Na⁻⁻⁻H⁺ antiport inhibition with 10⁻⁵ mol/L amiloride on the action of NH₄Cl.

**Experiment 3: Possible Contribution of Vasocostrictive Prostanoids and Superoxide to the Effect of NH₄Cl**

We examined the effect of treatments (20 minutes) with the PLA₂ inhibitor quinacrine (10⁻³ mol/L), cyclooxygenase inhibitor indomethacin (10⁻⁴ mol/L), prostaglandin H₁ (PGH₁)/ thromboxane A₂ (TXA₂) receptor antagonist S1452 (3×10⁻⁴ mol/L), TXA₂ synthase inhibitor dazmegrel (10⁻⁷ mol/L), and 5-lipoxygenase inhibitor AA861 (10⁻⁵ mol/L) on the action of NH₄Cl. Acetylcholine relaxation was also accomplished with treatment (10 minutes) with the superoxide scavenger superoxide dismutase (SOD, 100 U/mL). In addition, we examined the effect of the combination of SOD and indomethacin on acetylcholine relaxation.

**Experiment 4: Effect of Inhibited Nitric Oxide Generation on the Action of NH₄Cl**

Nitric oxide generation was abolished by 3×10⁻⁴ mol/L N⁰-nitro-l-arginine methyl ester (L-NAME). Ten minutes after L-NAME treatment, acetylcholine relaxation was accomplished in rat aorta with or without NH₄Cl. Also, we examined the effect of L-NAME plus 10⁻⁷ mol/L indomethacin.

**Drugs**

NH₄Cl, nigericin, amiloride, quinacrine, and L-NAME were purchased from Sigma Chemical Co; norepinephrine from Sankyo Pharmaceutical Co; acetylcholine and indomethacin from Wako Chemical Industries; and nitroglycerin from Nihon-Kayaku. The PGH₁/TXA₂ receptor antagonist S1452 was a gift from Shionogi, the TXA₂ synthase inhibitor dazmegrel from Pfizer, and the 5-lipoxygenase inhibitor AA861 from Takeda. Nigericin, amiloride, quinacrine, indomethacin, S1452, and AA861 were dissolved in ethanol. Dazmegrel was dissolved in 0.1 mol/L NaOH, and pH was adjusted to 8.5 with 0.1 mol/L HCl. Other drugs were dissolved in saline. Five microliters of each solution was added to treated aorta, and 5 μL of each vehicle (ethanol, 0.1 mol/L NaOH/0.1 mol/L HCl [pH 8.5], or saline) to untreated aorta (each vehicle did not affect acetylcholine relaxation; see "Results").

**Statistical Analysis**

Data are expressed as mean±SEM. The maximal relaxation by acetylcholine (Fmax, expressed as percentage of the level of norepinephrine-precontracted tension) and the acetylcholine concentration that produced a half-maximal relaxation (pD₂, expressed as minus logarithm of the concentration [mol/L]) were used for statistical comparison. Statistical analysis for Fmax and pD₂ was done by Student's t test (experiment 1; comparison between two [control and treated] groups) and by one-way (experiments 1 through 4) ANOVA and subsequent Tukey method. Values of P<.05 were considered significant.

**Results**

**Experiment 1: Effect of Changes in pH on Acetylcholine Relaxation**

Ten and 20 mmol/L NH₄Cl increased resting vascular tone slightly (3, 5, 10, and 20 mmol/L: not determined, 3±2%, 9±3%, and 13±4% of KCl contraction) and potentiated norepinephrine contraction (Fig 1). In contrast, 3 and 5 mmol/L NH₄Cl did not significantly change resting vascular tone and norepinephrine contraction. As shown in Table 1 and Fig 2, 3 mmol/L NH₄Cl treatment attenuated acetylcholine relaxation. The removal of NH₄Cl restored acetylcholine relaxation to control levels. Also, nigericin alone did not affect acetylcholine relaxation. In contrast to acetylcholine relaxation, NH₄Cl did not change nitroglycerin relaxation. Acetylcholine did not relax aortic rings without endothelium in control or NH₄Cl-added media.
TABLE 1. Effect of Changes In Intracellular pH on Acetylcholine Relaxation In Rat Aorta Precontracted by 3x10^-7 mol/L Norepinephrine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Without Treatment</th>
<th>With Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl (3 mmol/L)</td>
<td>8 92.7±2.0 7.40±0.07</td>
<td>7 74.5±4.0* 6.52±0.08*</td>
</tr>
<tr>
<td>Removal of NH₄Cl</td>
<td>5 93.4±0.8 7.21±0.05</td>
<td>5 91.6±1.8 7.22±0.06</td>
</tr>
<tr>
<td>Nigericin (10^-6 mol/L)</td>
<td>7 96.8±1.3 7.39±0.05</td>
<td>6 92.4±2.5 7.40±0.07</td>
</tr>
<tr>
<td>NTG relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl (3 mmol/L)</td>
<td>6 96.0±0.9 7.97±0.04</td>
<td>6 95.0±1.8 7.89±0.14</td>
</tr>
</tbody>
</table>

E_{max} indicates maximal relaxation by acetylcholine, expressed as percentage of precontracted levels of norepinephrine; pD₂, acetylcholine concentration that produced half-maximal relaxation, expressed as negative logarithm of concentration (mol/L); ACh, acetylcholine; and NTG, nitroglycerin. Values are mean±SEM.

*P<.05 compared with control.

Experiment 2: Time-Dependent Effect of NH₄Cl Treatment on Acetylcholine Relaxation

Acetylcholine relaxation was attenuated more by the 0-minute treatment of NH₄Cl than by the 10-minute treatment (Table 2). Nigericin inhibited the attenuating effect of both 0- and 10-minute treatments of NH₄Cl similarly (Fig 3). Acetylcholine relaxation was not affected by amiloride in control medium. Amiloride abolished the effect of the 10-minute treatment of NH₄Cl, but did not significantly change that of the 0-minute NH₄Cl treatment.

Experiment 3: Possible Contribution of Vasoconstrictive Prostanoids and Superoxide to the Effect of NH₄Cl

Quinacrine, indomethacin, S1452, dazmegrel, and AA861 did not change acetylcholine relaxation in control medium (Table 3). However, the impaired acetylcholine relaxation by NH₄Cl was reversed by quinacrine, indomethacin, S1452, and dazmegrel. In contrast, AA861 did not normalize but rather slightly enhanced the inhibitory effect of NH₄Cl. SOD did not affect E_{max} of acetylcholine relaxation in control medium but slightly enhanced pD₂. SOD inhibited the attenuation of acetylcholine relaxation by NH₄Cl. Indomethacin plus SOD also normalized the effect of NH₄Cl. In aortic rings with NH₄Cl plus the combined treatments, acetylcholine relaxation did not differ from that in rings with NH₄Cl plus either SOD or indomethacin.

Experiment 4: Effect of Inhibited Nitric Oxide Generation on the Action of NH₄Cl

L-NAME abolished acetylcholine relaxation in aorta with NH₄Cl treatment (Fig 4). In contrast, acetylcholine constricted aortic rings treated with NH₄Cl plus L-NAME (E_{max}, 19.6±5.1%; pD₂, 7.41±0.30). Indomethacin reversed the effect of NH₄Cl in L-NAME-treated aorta.

Discussion

In the present study, NH₄Cl treatment, which increases pH,_, attenuated acetylcholine-induced relaxation in rat aorta. The H⁺ ionophore nigericin, which increases intracellular H⁺ concentration and blocks NH₄Cl-induced intracellular alkalinization, normalized attenuation of acetylcholine relaxation. In addition, the removal of NH₄Cl from medium, which causes abrupt intracellular acidification despite slow extrusion of NH₄⁺ from cells, did not affect acetylcholine relaxation. Thus, intracellular H⁺ rather than intracellular NH₄⁺ may be critical in the attenuating effect of NH₄Cl in acetylcholine relaxation. This hypothesis is supported by the present finding that the effect of NH₄Cl disappeared in a time-dependent fashion (Table 2). When NH₄Cl was added extracellularly, NH₄⁺ rapidly moves into intracellular spaces and binds H⁺, resulting in intracellular alkalinization, in vascular smooth muscle and endothelial cells. On the other hand, NH₄⁺ slowly enters into the cell and releases H⁺, leading to a gradual normalization of pH. Thus, NH₄Cl-induced attenua-
Acetylcholine relaxation was performed after 10 minutes of

TABLE 2. Effect of Different Treatment Periods of 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>0-Minute (n=6)</th>
<th>10-Minute (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emax (nM)</td>
<td>93.5±2.1</td>
<td>66.1±5.5*</td>
<td>79.1±2.8*</td>
</tr>
<tr>
<td>pD2</td>
<td>7.35±0.04</td>
<td>6.50±0.17*</td>
<td>6.90±0.20†</td>
</tr>
</tbody>
</table>

E<sub>max</sub> indicates maximal relaxation by acetylcholine, expressed as percentage of precontracted levels of norepinephrine; pD<sub>2</sub>, acetylcholine concentration that produced half-maximal relaxation, expressed as negative logarithm of concentration (mol/L). In 0-minute treatment, cumulative acetylcholine relaxation was started immediately after NH<sub>4</sub>Cl addition; in 10-minute treatment, acetylcholine relaxation was performed after 10 minutes of NH<sub>4</sub>Cl treatment. Values are mean±SEM.

*P<.05 compared with control.
†P<.05 compared with 0-minute treatment.

Thus, acetylcholine relaxation may follow pH alteration with NH<sub>4</sub>Cl treatment. Moreover, amiloride normalized the suppressive effect of the 10-minute treatment of NH<sub>4</sub>Cl but not the 0-minute treatment, although nigericin inhibited NH<sub>4</sub>Cl-induced attenuation irrespective of the treatment period. Na<sup>+</sup>-H<sup>+</sup> antiport, which extrudes H<sup>+</sup>, may delay the amelioration of NH<sub>4</sub>Cl-induced alkalinization.<sup>16,17</sup> Thus, the blockade of Na<sup>+</sup>-H<sup>+</sup> antiport may facilitate the return of pH, to basal levels and accelerate the reversal of acetylcholine relaxation from NH<sub>4</sub>Cl-induced attenuation.

Although intracellular alkalinization induced by a high dose (>10 mmol/L) of NH<sub>4</sub>Cl affected vascular tone directly, the direct vascular effects may not contribute to our results. Previous studies indicated that a relatively high dose (>10 mmol/L) potentiated vasoconstriction of KCl<sup>15,16</sup> and phenylephrine<sup>16</sup> in rat aorta and that a high dose (>20 mmol/L) of NH<sub>4</sub>Cl produced relaxation.<sup>14</sup> In contrast, 3 mmol/L NH<sub>4</sub>Cl did not affect basal tension of aorta and norepinephrine-induced contraction (see "Results" and Fig 1). Moreover, nitroglycerin relaxation was not affected by NH<sub>4</sub>Cl treatment. Acetylcholine did not relax denuded aorta in control medium or medium to which NH<sub>4</sub>Cl had been added. Thus, vascular smooth muscle cells may not participate in the attenuation of acetylcholine relaxation by small doses (3 mmol/L) of NH<sub>4</sub>Cl in rat aorta. High CO<sub>2</sub> tension suppressed norepinephrine-induced contraction in rat aorta, possibly with endothelial modulation.<sup>5</sup> Our recent study<sup>6</sup> suggested that acetylcholine relaxation was modified by altered activity of Na<sup>+</sup>-H<sup>+</sup> antiport. Thus, NH<sub>4</sub>Cl-induced impairment of acetylcholine relaxation may be mediated through endothelial function.

The optimal pH of PLA<sub>2</sub> is neutral to alkaline: in human platelets, epinephrine-evoked stimulation of PLA<sub>2</sub> activity was inhibited by blockade of Na<sup>+</sup>-H<sup>+</sup> antiport and reversed by intracellular alkalinization with methylamine.<sup>11,12</sup> Vasoactive substances that cause intracellular alkalinization have been reported to produce prostaglandins.<sup>16,17</sup> In the present study, inhibition of PLA<sub>2</sub> (quinacrine), cyclooxygenase (indomethacin), and TXA<sub>2</sub> synthase (dazmegrel) and antagonism of the PGH<sub>2</sub>/TXA<sub>2</sub> receptor (S1452) normalized the attenuated acetylcholine relaxation in rat aorta treated with 3 mmol/L NH<sub>4</sub>Cl. Because 3 mmol/L NH<sub>4</sub>Cl attenuated acetylcholine relaxation but did not affect resting tension or norepinephrine contraction, TXA<sub>2</sub> may be stimulated only in the presence of acetylcholine and may be a possible mediator of the inhibitory effect of intracellular alkalinization by NH<sub>4</sub>Cl treatment on acetylcholine relaxation. This hypothesis is supported by the fact that acetylcholine constricted
TABLE 3. Changes In \( \text{NH}_4 \text{Cl} \)-Induced Attenuation of Acetylcholine-Induced Relaxation by Several Drugs In Rat Aorta Precontracted by \( 3 \times 10^{-7} \text{ mol/L} \) Norepinephrine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th></th>
<th>( \text{NH}_4 \text{Cl} )</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Quinacrine} \ (10^{-5} \text{ mol/L}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>99.0( \pm )0.9</td>
<td>91.5( \pm )3.0</td>
<td>69.2( \pm )3.3*</td>
<td>96.3( \pm )2.1†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.56( \pm )0.06</td>
<td>7.09( \pm )0.27</td>
<td>6.39( \pm )0.05*</td>
<td>7.39( \pm )0.03†</td>
</tr>
<tr>
<td>( \text{Indomethacin} \ (10^{-5} \text{ mol/L}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>93.9( \pm )2.3</td>
<td>93.3( \pm )2.2</td>
<td>71.9( \pm )4.0*</td>
<td>93.8( \pm )2.5†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.42( \pm )0.13</td>
<td>7.28( \pm )0.07</td>
<td>6.47( \pm )0.08*</td>
<td>7.49( \pm )0.05†</td>
</tr>
<tr>
<td>( \text{S145} \ (3 \times 10^{-8} \text{ mol/L}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>97.6( \pm )1.3</td>
<td>91.8( \pm )2.8</td>
<td>74.4( \pm )3.5*</td>
<td>91.7( \pm )2.3†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.44( \pm )0.06</td>
<td>7.24( \pm )0.11</td>
<td>6.49( \pm )0.09*</td>
<td>7.05( \pm )0.07†</td>
</tr>
<tr>
<td>( \text{Dazmegrel} \ (10^{-5} \text{ mol/L}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>96.8( \pm )1.5</td>
<td>94.3( \pm )1.4</td>
<td>74.5( \pm )4.0*</td>
<td>89.5( \pm )3.3†</td>
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<tr>
<td>( pD_2 )</td>
<td>7.41( \pm )0.10</td>
<td>7.77( \pm )0.10</td>
<td>6.52( \pm )0.08*</td>
<td>7.43( \pm )0.33†</td>
</tr>
<tr>
<td>( \text{AA861} \ (10^{-5} \text{ mol/L}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>95.7( \pm )0.4</td>
<td>89.3( \pm )0.6</td>
<td>72.2( \pm )1.5*</td>
<td>55.3( \pm )2.0†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.48( \pm )0.02</td>
<td>7.09( \pm )0.27</td>
<td>6.52( \pm )0.03*</td>
<td>6.47( \pm )0.04*</td>
</tr>
<tr>
<td>( \text{SOD} \ (100 \text{ U/mL}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>91.2( \pm )1.9</td>
<td>92.9( \pm )0.8</td>
<td>73.8( \pm )3.5*</td>
<td>95.2( \pm )1.9†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.38( \pm )0.07</td>
<td>7.71( \pm )0.09*</td>
<td>6.49( \pm )0.08*</td>
<td>7.34( \pm )0.02†</td>
</tr>
<tr>
<td>( \text{Indomethacin+SOD} \ (10^{-5} \text{ mol/L}+100 \text{ U/mL}) )</td>
<td>Without Treatment</td>
<td>With Treatment</td>
<td>Without Treatment</td>
<td>With Treatment</td>
</tr>
<tr>
<td>( n )</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>( E_{\text{max}} )</td>
<td>93.5( \pm )1.9</td>
<td>94.7( \pm )3.6</td>
<td>74.0( \pm )5.2*</td>
<td>94.1( \pm )2.8†</td>
</tr>
<tr>
<td>( pD_2 )</td>
<td>7.19( \pm )0.09</td>
<td>7.41( \pm )0.11</td>
<td>6.60( \pm )0.09*</td>
<td>7.27( \pm )0.11†</td>
</tr>
</tbody>
</table>

* indicates maximal relaxation by acetylcholine, expressed as percentage of precontracted levels of norepinephrine; \( pD_2 \), acetylcholine concentration that produced half-maximal relaxation, expressed as negative logarithm of concentration (mol/L); and SOD, superoxide dismutase. Values are mean\( \pm \)SEM.

*\( P<.05 \) compared with control.
†\( P<.05 \) compared with \( \text{NH}_4 \text{Cl} \) treatment alone.

\( \text{NH}_4 \text{Cl} \)-treated aorta with the blockade of nitric oxide production by L-NAME and that simultaneous indomethacin treatment abolished this contraction. In contrast, the blockade of 5-lipoxygenase (AA861) did not ameliorate the attenuating effect of \( \text{NH}_4 \text{Cl} \) on acetylcholine relaxation but rather enhanced it. Thus, leukotrienes may not contribute to the attenuating effect of \( \text{NH}_4 \text{Cl} \) on acetylcholine relaxation but compensate it, because leukotriene \( \text{D}_4 \) relaxed blood vessels.¹⁸

Increased pH, has been indicated to be accompanied by increased intracellular \( \text{Ca}^{2+} \) (\( \text{Ca}^{2+} \)\text{),¹⁹ which \( \text{PLA}_{2} \) requires in synthesizing arachidonic acid.²⁰ In bovine aortic endothelial cells, intracellular alkalinization by \( \text{NH}_4 \text{Cl} \) leads to increased \( \text{Ca}^{2+} \).¹⁷ Also, \( \text{Ca}^{2+} \) was increased in human endothelial cells with alkalinization by either \( \text{NH}_4 \text{Cl} \) or monensin.²¹ Thus, intracellular alkalinization may not only enhance the affinity of \( \text{PLA}_{2} \) to \( \text{Ca}^{2+} \), but also increase \( \text{Ca}^{2+} \), itself. Both enhanced affinity and increased \( \text{Ca}^{2+} \) facilitate the production of arachidonic acid, resulting in increased vasoconstrictor prostaglandins. In fact, the role of both pH and \( \text{Ca}^{2+} \), was suggested in prostanoid release from Kupffer cells after hypoxia-reoxygenation.²² Intracellular alkalinization induced by 3 mmol/L \( \text{NH}_4 \text{Cl} \) might not elevate \( \text{Ca}^{2+} \) sufficiently to stimulate basal activity of \( \text{PLA}_{2} \) but may increase the affinity of \( \text{PLA}_{2} \) to enhance the response to acetylcholine-induced rise in \( \text{Ca}^{2+} \), because it affected vascular tone only in the presence of acetylcholine.

In addition to \( \text{TXA}_{2} \), the present results suggest that superoxide radicals may mediate the effect of \( \text{NH}_4 \text{Cl} \) because SOD restored the effect. The combination of extracellular alkalinization and \( \text{N}-\text{formyl-methionyl-leucyl-phenylalanine} \) increased both pH and the generation of superoxide in human neutrophils, but extracellular alkalinization alone did not affect either, suggesting that the intracellular alkalinization should be
critical in enhanced superoxide production. Also, endothelin, which increased pH, produced superoxide in human alveolar macrophages. Superoxide anion has also been indicated to be generated in endothelial cells, so it may act as a contracting factor. In the present study, there was no difference between acetylcholine relaxation in aortas treated with indomethacin or indomethacin plus SOD, compatible with the hypothesis that superoxide is generated from the cyclooxygenase pathway, as suggested previously. A recent report suggested that PGH₂ inhibited acetylcholine relaxation by formation of superoxide radicals in aortic endothelial cells of rabbit. The present results suggest that the most plausible prostanoid, which inhibited acetylcholine relaxation with intracellular alkalinization, may be TXA₂, rather than PGH₂ but that PGH₂ could generate superoxide and attenuate acetylcholine relaxation. However, superoxide also may be generated by other pathways, such as xanthine oxidase, cytochrome P-450, or various cellular enzymes. Thus, further investigations are required to clarify the precise mechanism that superoxide contributes to the effect of intracellular alkalinization.

In conclusion, intracellular alkalinization by NH₄Cl attenuated acetylcholine-induced relaxation in rat aorta. This effect of NH₄Cl was reversed by suppressed TXA₂ generation, its receptor antagonist, or SOD. Thus, the attenuation of acetylcholine-induced relaxation by intracellular alkalinization may be through increased TXA₂ and superoxide production in vascular endothelial cells.

References


Inhibitory effect of ammonium chloride on acetylcholine-induced relaxation.
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Hypertension. 1994;24:189-194
doi: 10.1161/01.HYP.24.2.189

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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