

Effect of sensitization on membrane ion fluxes & intracellular calcium in guineapigs

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Background & objectives: The biochemical mechanisms underlying the development of sensitization-induced airway hyperresponsiveness (AHR) in asthma are poorly defined. Alterations in the regulation of intracellular calcium may play an important role in its pathogenesis. We carried out this study to see the effect of sensitization with ovalbumin on membrane ion fluxes and intracellular calcium in a guinea pig model.

Methods: Airway reactivity to inhaled histamine was measured initially and after sensitization with ovalbumin in 28 guineapigs. Intracellular calcium $[Ca^{2+}]_i$ was measured in tracheal smooth muscle cells and peripheral leukocytes using fluorescent dye FURA 2AM. Calcium and sodium ion influx across the cell membrane was measured in leukocytes. Ouabain-sensitive Rubidium (^{86}Rb) influx was measured in tracheal smooth muscles cells. The activities of Na^+ , K^+ ATPase and Ca^{2+} ATPase were measured in tracheal smooth muscle cells. Lipid peroxides were measured in plasma.

Results: Airway responsiveness was significantly ($P<0.001$) increased after sensitization along with an increase in $[Ca^{2+}]_i$ levels in leukocytes and tracheal smooth muscle cells, higher rates of ^{45}Ca and ^{22}Na influx in leukocytes and higher ^{86}Rb influx rates in tracheal smooth muscle cells, and increased levels of lipid peroxides in plasma.

Interpretation & conclusion: In guineapig model of asthma sensitization to allergen increased the membrane permeability to calcium and sodium, and intracellular calcium levels. These alterations may play a role in the pathogenesis of airway hyper-responsiveness following sensitization.

Key words Airway hyperresponsiveness - asthma - calcium influx - intracellular calcium - intracellular sodium-sensitization - sodium influx - smooth muscle

The mechanisms underlying airway hyperresponsiveness (AHR) in asthma are poorly defined. A major reason for this is that it is usually

difficult to obtain airway smooth muscle of asthmatics for *in vitro* studies. Sensitization with antigen has been shown to induce AHR, both *in vivo*¹ and *in vitro*², in

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animal models. Models such as sensitized guineapigs may therefore be suitable for investigating the biochemical abnormalities associated with the process of sensitization. Souhrada and Souhrada^{2,3} have provided *in vitro* evidence that both active and passive sensitization produce changes in the membrane potential of airway smooth muscle associated with altered membrane permeability leading to increased influx of Na^+ and Ca^{2+} , and, an exaggerated contractile response to histamine. Perpina *et al*⁴ have reported an increased uptake of ^{45}Ca in tracheal smooth muscle from sensitized guineapigs. A defect in the regulation of intracellular calcium $[\text{Ca}^{2+}]_i$ has been proposed in asthma^{5,6}. Previous studies from our laboratory⁶ have shown a direct correlation between *in vivo* airway reactivity in guinea pigs and the activities of enzymes involved in the regulation of intracellular calcium and sodium, Ca^{2+} and Na^+ , K^+ ATPases⁶. We have also reported increased levels of intracellular calcium in leukocytes of asthma patients that were directly correlated with the severity of disease⁷. Orlov *et al*⁸ reported increased ^{86}Rb uptake and higher intracellular sodium levels in erythrocytes of asthmatic patients. Thus, there is some evidence that transport and regulation of intracellular calcium and sodium is altered in asthmatics. The influx of calcium and sodium, $[\text{Ca}^{2+}]_i$ levels and the activities of the ATPases have however not been measured directly in airway smooth muscle cells. Whether the increase in AHR known to occur after sensitization is associated with alterations in transport and regulation of these intracellular cations needs to be studied. The present study was thus carried out with this objective in a guineapig model of asthma induced by sensitization with ovalbumin (OVA).

Material & Methods

Healthy male guineapigs weighing 250-400 g were used. These were housed in climate-controlled animal quarters in the animal house of the Vallabhbhai Patel Chest Institute and were given water and food *ad libitum*. Approval for the study was obtained from the Board of Research Studies in Medical Sciences, University of Delhi. Procedures followed were in accordance with the ethical standards.

Histamine, Tris, [N-(2 Hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)] (HEPES), ethylene

glycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), ionomycin, bovine serum albumin and ouabain were obtained from Sigma, USA. Fura-2AM was bought from Molecular Probes Inc., USA. All other chemicals and reagents were locally available, laboratory grade chemicals.

Baseline specific airways conductance (sGaw) and airway responsiveness to histamine were measured using a non invasive body plethysmographic technique⁹. Briefly, box pressure and airflow was measured using Validyne (USA) PM 18 differential transducers and the signal was amplified with CD12 carrier demodulators (Validyne, USA). The box pressure signal was fed on the X channel and airflows on the Y channel of Hewlett-Packard (USA) oscilloscope to obtain an X-Y plot. The $\tan \theta$ of the rising limb of the tracing was used to compute sGaw⁹. The dose of histamine that produced a 35 per cent fall in sGaw was determined and was labeled as ED_{35} histamine. This represented the airway reactivity of the animal and was measured before and 4-6 wk after sensitization. The guineapigs were sensitized with 100mg aluminium hydroxide and 100 μg OVA per ml of normal saline as described by Santing *et al*¹⁰. Half the dose of ovalbumin was given intraperitoneally and the remaining was injected in equal amounts at the sites of lymph nodes - cervical, axillary and inguinal. This sensitization protocol has been shown to favour an IgE-mediated response¹⁰ and the sensitization occurs by end of 4 wk. A control group of non-sensitized animals was also included.

The animals were anaesthetized with an intraperitoneal (ip) injection of pentothal sodium (50mg/kg) and blood was drawn into heparinized syringes (10U/ml blood) directly from the heart. Isolation of leukocytes was done by dextran sedimentation. The pellet consisted of solely of morphologically intact leukocytes. Cell viability, as assessed by trypan blue exclusion¹¹ was found to be > 95 per cent. The tracheal smooth muscle cells were isolated by enzymatic digestion¹².

Measurement of $[\text{Ca}^{2+}]_i$: $[\text{Ca}^{2+}]_i$ was measured by using the cell membrane permeant fluorescent dye FURA 2AM (Molecular Probes, USA)¹³. Two million leukocytes, or tracheal smooth muscle cells, were loaded with FURA 2AM by incubation with 2 μl of

1 mmol stock of dye in dimethyl sulphoxide (DMSO) to a final concentration of 2 μ mol for 45 min at 37°C. The cells were then washed thrice with HEPES buffer by centrifuging at 400 g for 2 min. Fluorescence was measured in 2 ml of cell suspension in a Shimadzu RF-5000 spectrofluorometer (Japan) by dual excitation at 340 and 380 nm and emission at 510 nm. The $[Ca^{2+}]_i$ was calculated by the ratio method using the equation: $[Ca^{2+}]_i = K_d (R - R_{min}) / (R - R_{max}) \times Sf_2 / Sf_1$ where K_d is dissociation constant of FURA-2AM for (Ca^{2+}) taken as 224 nmol at 37°C, R is ratio of fluorescence for FURA 2AM at the two excitation wavelengths *i.e.*, F_{340}/F_{380} , R_{max} is ratio of fluorescence in the presence of excess of calcium obtained by lysing the cells with 10 μ mol ionomycin (Sigma, USA), R_{min} is ratio of fluorescence in the presence of minimal calcium obtained by lysing the cells and then chelating all the Ca^{2+} with 0.5 M EGTA, Sf_2 is fluorescence of Ca^{2+} free form of FURA 2AM at 380nm excitation wavelength and Sf_1 is fluorescence of Ca^{2+} bound form of FURA 2AM at 380 nm excitation wavelength. The auto-fluorescence of the leukocytes was subtracted from the observed values before the ratios were calculated.

Sodium-potassium and calcium ATPase activity: The tracheae were minced into small pieces and homogenized using a ground glass homogenizer. The homogenate was used for ATPase assay that was measured as described by Schmalzing and Kushera¹⁴. Total protein in the homogenate was estimated with bovine serum albumin (BSA) as the standard¹⁵. The inorganic phosphorous (P_i) liberated by the ATPase activity was estimated by spectrophotometric method¹⁶. The results were expressed as μ g P_i liberated from ATP/ mg protein/ 30 min at 37°C.

Measurement of ^{86}Rb uptake: Basal $Na^+ K^+$ ATPase activity was measured by estimating transmembrane movement of ^{86}Rb , which mimics K^+ movement, in the presence or absence of ouabain, a specific inhibitor of $Na^+ K^+$ ATPase as described by Oh *et al*¹⁷. Tracheal strips, 2 to 3 mm wide, were equilibrated in the basal Krebs's Ringer medium containing 150 mM NaCl, 0.25 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2HPO_4 , 5 mM Na_2HPO_4 and 10 mM dextrose, with or without 1 mM ouabain. The reaction was started by adding 8.3 μ M Rb^{2+} (Molecular Probes, USA) equivalent to

approximately 1 μ Ci/ μ l. Uptake was arrested by washing the strips 5 times with ice cold basal medium. The strips were solubilized with 0.1 per cent triton and an aliquot from each sample was used for measurement of radioactivity, while the remaining lysate was used for protein assay. Basal $Na^+ K^+$ ATPase activity was determined by calculating the difference between 'total Rb^+ uptake' in the absence of ouabain and 'ouabain-resistant' uptake. K^+ uptake was calculated by accounting for the relative amount of cold K^+ and labeled Rb^+ in the intracellular space. Assuming the energy coupling of one molecule of ATP for each molecule of K^+ transported, the results were expressed as μ g ATP hydrolysed (or P_i liberated)/mg protein/30 min at 37°C.

Measurement of Na^+ and Ca^{2+} influx: Sodium and calcium ion uptake was measured as described by Simcowitz *et al*¹⁸. Isolated leukocytes were incubated at 37°C in the presence of 0.4 μ Ci ^{22}Na (Molecular Probes, USA) or ^{45}Ca (Molecular Probes, USA) for 7.5 min. The cells were then washed twice in buffer and finally separated from the aqueous phase by centrifugation over a layer of di-butyl phthalate at 10500g. The cells were finally lysed with 0.1 per cent triton X-100 in 0.1N sodium hydroxide at 51°C for 1 h and the counts in the lysate were taken on a Beckman β -scintillation counter (USA). The ion influx was expressed as pmoles of ion/ 10^6 -cells/ min.

Lipid peroxidation was estimated as thiobarbituric acid reactivity of malonaldehyde, an end product of fatty acid peroxidation¹⁹.

Statistical analysis: The data were expressed in mean $\pm$ SEM and analysed using SPSS 9.0 for Windows and Graphpad Prism 2.01. For comparison of paired and unpaired variables, Students t-test (paired/unpaired) was used for normally distributed data and Wilcoxon Signed rank test/Mann Whitney U test were used if distribution was not normal.

Results

The specific airways conductance was measured in 49 non-sensitized and 28 sensitized animals. The baseline mean sGaw in the two groups was 0.17 ± 0.008 and 0.16 ± 0.008 sec⁻¹cm H₂O⁻¹, respectively ($P>0.05$). In sensitized animals, the post-sensitization

sGaw was not different from the pre-sensitization value ($0.14 \pm 0.03/\text{sec}/\text{cm H}_2\text{O}$).

Effect of sensitization on airway reactivity: Sensitization induced a significant increase in airway reactivity (Fig.). The median ED_{35} histamine was 2.5 mg/ml before sensitization and 0.89 mg/ml after sensitization ($P < 0.001$). Before sensitization, 10 guineapigs were reactive and 18 were non-reactive. After sensitization, 15 of the non-reactive ones became reactive. In four animals reactive to histamine even before sensitization, sensitization did not alter the reactivity. The remaining animals responded with a greater than 2-fold increase in airway sensitivity to histamine. Biochemical studies were carried out in animals that became reactive or had increased reactivity to histamine after sensitization. A positive wheal response (>3 mm) to intradermal injection of ovalbumin into the earlobe skin confirmed sensitization.

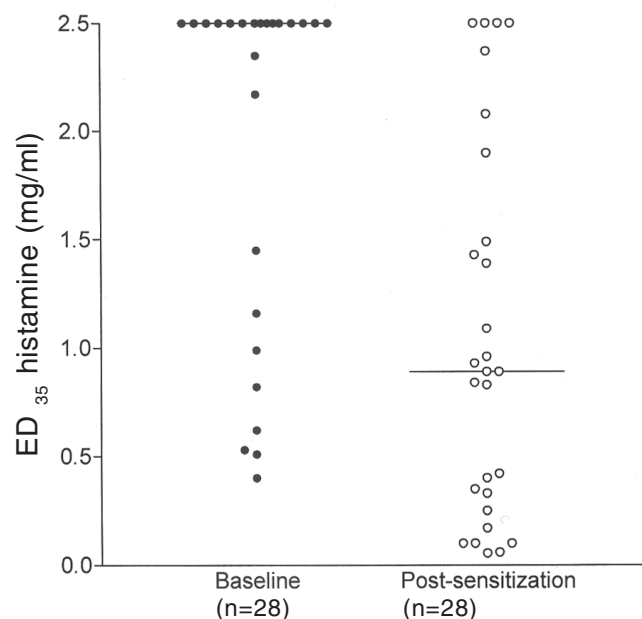


Fig. Airway reactivity (ED_{35} histamine, mg/ml) at baseline and after sensitization. The horizontal line represents the median.

Effect of sensitization on leukocyte and tracheal (Ca^{2+})i: Sensitized animals ($n=11$) were found to have significantly greater levels of $[\text{Ca}^{2+}]_i$ in isolated tracheal smooth muscle cells as compared to non-sensitized animals ($n=18$) (623.59 ± 139.01 and 149.47 ± 27.1 nmol/l respectively, $P < 0.01$). Similarly, leukocyte (Ca^{2+})i was significantly greater in sensitized animals than in the non-sensitized animals (366.1 ± 82.34 and 184.91 ± 29.18 nmol/l respectively, $P < 0.05$).

Effect of sensitization on maximal tracheal ATPase activities: The ATPase activities were expressed as $\mu\text{g P}_i/\text{mg protein}/30$ min. There was no significant difference between sensitized ($n=14$) and non-sensitized ($n=18$) guineapigs in the tracheal Ca^{2+} ATPase activity (3.57 ± 0.63 and 2.57 ± 0.52 , respectively, $P > 0.05$) or in the Na^+ , K^+ ATPase activity (5.02 ± 0.75 and 4.51 ± 1.0 , respectively, $P > 0.05$).

Effect of sensitization on rubidium (^{86}Rb) uptake: Rubidium (^{86}Rb) uptake studies were carried out in intact tracheal smooth muscle cells from 10 sensitized guineapigs and compared with 5 non sensitized reactive animals. The sensitized animals were found to have a significantly greater rate of ^{86}Rb uptake (1.0 ± 0.23 $\mu\text{g ATP hydrolysed (or P}_i \text{ liberated)}/\text{mg protein}/30\text{min}$) as compared to non sensitized guinea-pigs (0.65 ± 0.21 units) ($P < 0.05$).

Effect of sensitization on the rate of calcium (^{45}Ca) influx: The rate of ^{45}Ca influx was determined in both intact tracheal smooth muscle cells and leukocytes. No significant difference was observed in the influx in tracheal cells of non sensitized and sensitized animals ($n=12$) (0.075 ± 0.022 and 0.047 ± 0.08 pmoles/mg tissue/min respectively). However, ^{45}Ca influx was significantly higher in leukocytes of sensitized animals ($n=4$) as compared to the non sensitized ($n=6$) animals (7.0 ± 1.70 and 2.27 ± 0.56 units respectively, $P < 0.05$).

Effect of sensitization on the rate of sodium (^{22}Na) influx: The rate of ^{22}Na influx was determined in both intact tracheal smooth muscle cells and leukocytes. Sodium influx in tracheal smooth muscle cells of sensitized ($n=9$) and non-sensitized ($n=6$) animals was not significantly different (2.13 ± 0.66 and 1.75 ± 0.55 pmoles/mg tissue/min respectively). In the

leukocytes, these rates were 951.88 ± 74.69 pmoles/ 10^6 cells/min in sensitized animals ($n=4$) and 609.8 ± 69.36 pmoles/ 10^6 cells/min in non sensitized animals ($n=6$). The difference was statistically significant ($P<0.05$).

There was no correlation between post-sensitization airway reactivity and the ion fluxes. The correlation coefficients were as follows: ED_{35} histamine and ^{86}Rb influx ($r=0.011$, $P>0.05$), ED_{35} histamine and ^{45}Ca influx ($r=0.083$, $P>0.05$), ED_{35} histamine and ^{22}Na influx ($r= -0.51$, $P>0.05$).

Effect of sensitization on lipid peroxide levels: Lipid peroxides were measured in the plasma of 10 animals each in the sensitized and non sensitized groups. The levels in the non sensitized guineapigs were 0.31 ± 0.05 nmol TBARS/ml. In contrast, sensitized guineapigs had significantly higher levels of lipid peroxides (0.54 ± 0.09 nmol TBARS/ml plasma, $P<0.05$).

Discussion

The findings of the present study showed that active sensitization with allergen altered the regulation of intracellular calcium, possibly by an effect on the cell membrane. Guinea pigs sensitized to allergen showed increased *in vivo* airway responsiveness to inhaled histamine. The sensitized animals had significantly greater levels of intracellular calcium in isolated tracheal smooth muscle cells and peripheral blood leukocytes as compared to the non sensitized ones. Further, the sensitized animals showed evidence of increased membrane permeability to calcium and sodium ions as reflected in a higher influx of ^{45}Ca and ^{22}Na in leukocytes. Increased levels of lipid peroxides in plasma of sensitized guineapigs implying increased oxidative stress were observed.

The biochemical basis of AHR has not been defined although it has been recognized as the characteristic physiological abnormality in asthma. Souhrada and Souhrada^{2,3,20} have provided evidence that sensitization has an effect on the cellular mechanisms responsible for the maintenance of membrane potential and the regulation of excitation-contraction coupling in airway smooth muscle. They showed that *in vitro* passive sensitization altered membrane permeability for

sodium ions and induced a biphasic change in the resting membrane potential of isolated airway smooth muscle cells - a transient depolarization followed by a sustained hyperpolarization attributable to activation of the Na^+ pump. Presence of ouabain, a specific inhibitor of the Na^+ pump, and amiloride, a specific sodium entry blocker were shown to inhibit the contractile responses to antigen². Subsequently, the same workers suggested that the increase in Na^+ influx was associated with an increase in Ca^{2+} influx as the isometric force developed was also increased³. Perpina *et al*⁴ demonstrated the existence of hyperreactivity and altered ^{45}Ca movements *i.e.*, increased uptake and retention, in sensitized tracheal smooth muscle. Our observations corroborate the earlier indirect evidence of increased fluxes of Na^+ and Ca^{2+} after sensitization. In addition, we have documented that sensitization alters intracellular calcium homeostasis resulting in higher levels under resting conditions.

The relationship between Na^+ and Ca^{2+} influx is complex. Activation of calcium channels by depolarization was observed after receptor occupation in smooth muscle cells^{21,22}. Transmembrane sodium flux has also been implicated in the maintenance of cytosolic calcium ion levels²³. Release of calcium from intracellular stores by thapsigargin, a specific inhibitor of endoplasmic reticulum Ca^{2+} ATPase, has been shown to activate sodium influx in human platelets²⁴. The mechanism underlying the increased calcium and sodium fluxes across cell membranes is not known. Phorbol esters that act through stimulation of protein kinase C (PKC) were shown to cause a comparable change in both the resting membrane potential and the isometric force of isolated smooth muscle²⁵. Therefore, stimulation of PKC was implicated in responses to sensitization by inducing increased calcium influx²⁶. It was later shown that these changes were inhibited by inhibition of PKC²⁷. It is however unlikely that voltage-operated channels are involved in increased calcium influx, given the disappointing results obtained with calcium channel antagonists in asthma. Young *et al*²⁸ had shown that in alveolar macrophages, the Fc receptor is a ligand-dependent sodium channel. Occupancy of Fc receptors with specific antibodies results in an inward sodium current²⁹. Our findings are consistent with these observations.

The higher resting levels of $[Ca^{2+}]_i$ observed in the leukocytes and tracheal smooth muscle cells of sensitized animals in the present study are likely to be important in modulating airway reactivity as these would lower the threshold stimulus required for response. This may explain the AHR that occurs following active sensitization. This increase in $[Ca^{2+}]_i$ may have resulted from an influx from extracellular space demonstrated in the present study. Another possible mechanism is release from intracellular stores. Increased release of $[Ca^{2+}]_i$ from intracellular stores has been shown earlier in asthmatics³⁰.

Sensitization did not affect the activities of Ca^{2+} ATPase and Na^+ , K^+ ATPase. This contrasts with our earlier report of decreased activities of these enzymes in leukocytes of asthmatics⁷. However, the present model was different from clinical asthma. The animals were not challenged with antigen after sensitization as the aim was to study the biochemical changes due to sensitization alone. Another finding in the present study was increased influx rates of ^{86}Rb in sensitized animals. Rubidium is a congener of potassium and its influx rates are a reflection of the state of activation of the Na, K pump. Higher ^{86}Rb rates therefore suggest an increased activity of the Na, K pump after sensitization. Souhrada and Souhrada²⁰ had made similar inference from their experiments on smooth muscle contractility after sensitization. An increase in the Na, K pump activity is likely to have occurred as a response to increased intracellular Na^+ levels following increased influx of the ion. Although we have not measured intracellular sodium levels, an increased activity of the Na, K pump suggests that these levels were also increased. It may be pointed out that the measurement of Na^+ , K^+ ATPase activity as done in the present study reflects its maximal activity while the ^{86}Rb influx in resting cells reflects the current state of activation of the physiological counterpart of the enzyme, the Na, K pump.

We observed increased levels of lipid peroxides in plasma of sensitized guineapigs implying increased oxidative stress. Antigen challenge has been shown to enhance superoxide generation in sensitized guineapigs³¹. The role played by increased oxidative stress in modulation of cellular responses is not well established. Inflammatory mediators and oxidative stress have been reported to induce mobilization of

Ca^{2+} from both mitochondria and endoplasmic reticulum and it has been suggested that the alteration in $(Ca^{2+})_i$ homeostasis is a key early event in the generation of lethal cell injury due to oxidative stress³². Whether there is a link between the observation of increased oxidative stress and the alterations in intracellular calcium and sodium homeostasis requires further study.

In sensitized animals, the post-sensitization sGaw was not different from the presensitization value. sGaw and airway reactivity are two different measurements. sGaw reflect airway caliber at any time. It will decrease only after a sensitized animal is challenged with ovalbumin to induce an antigen-antibody reaction in the airways. This is similar to what happens in allergic asthmatics. If an allergic asthmatic is not exposed to an allergen, his lung function will be normal. As no challenge with antigen was given, the sGaw remained unchanged while airway reactivity increased due to sensitization.

In conclusion, the results of this study showed that sensitization to allergen increased the membrane permeability to calcium and sodium and resulted in higher intracellular calcium levels. These alterations may play a role in the pathogenesis of airway hyperresponsiveness following sensitization.

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