Polyamines in inflammation and their modulation by conventional anti-inflammatory drugs

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Received 23 January 2007; revised 12 April 2007

Significant increase in polyamines levels in inflamed tissue was observed in the experimental animal models of inflammation. Treatment with dexamethasone positively modulated the levels of polyamines whereas non-steroidal drugs, dicyclofenac and valdecoxib negatively modulated their levels.

Keywords: Dexamethasone, Diclofenac, Polyamines, Models of inflammation, Valdecoxib

The aliphatic polyamines, putrescine, spermidine and spermine are polycations existing in all living cells and have demonstrated their vital role in physiological functions. They stabilize anionic cell components such as membranes and nucleic acids (cell and cell organelles). Polyamines regulate gene expression, signal transduction, ion channel function, DNA and protein synthesis and proliferation and differentiation. They are scavengers of reactive oxygen species, thereby protecting DNA, proteins, and lipids from oxidative damage.

Polyamines have anti-inflammatory and anti-oxidant properties. In vitro studies have revealed that polyamines control the production of inflammatory mediators in several cell lines or tissues. Polyamines have been shown to be associated in gut and liver inflammation and therefore, significance of the levels of polyamines in liver during inflammation has been studied. However, the studies pertaining to the levels of putrescine, spermidine and spermine in the inflamed tissue and their likely role in inflammation is not clear. Inflammation is a proliferative process and proliferation involves increased DNA and protein synthesis, which in turn require elevated levels of polyamines. The determination of polyamines in the inflamed tissue will help to understand exact mechanisms at the healing site. Hence, the present study has been undertaken with the objectives of determining the polyamines content in the inflamed tissue of experimental rat models of inflammation. Further, an effort was made to correlate the effect of conventional anti-inflammatory drugs treatment on the polyamines levels in the edematous and granulomatus tissue and also to find out correlation between polyamines levels and anti-inflammatory activity. Finally, the significance of polyamines with the proliferative process and its possible application in anti-inflammatory drug screening.

Materials and Methods

Animals— Wistar strain rats (120-150 g) were purchased from the registered breeder, Bharath Sera Pvt Ltd., Mumbai, India. They were housed under good hygienic conditions in the departmental animal house. The animals were housed under standard conditions of temperature (24°C±1°C), RH (65 ± 10%), light (14 hr); dark (10 hr) cycle and fed with standard pellet food (Amrut Laboratory animal feed diet, Maharashtra, India) and water ad libitum.

Drugs, chemicals and reagents— Dexamethasone sodium was a gift sample from Halcyon Chemicals, India. Valdecoxib and dicyclofenic sodium were gift samples from Themis Laboratories, India. Dansyl chloride and Putrescine di-hydrochloride were purchased from Hi-Media, Mumbai, India. Spermidine and spermine were purchased from Sisco Laboratories, Mumbai, India. Freund’s adjuvant (complete) was a kind gift sample from Bharat Sera...
Vaccines, India. Carrageenin was purchased from Sigma-Aldrich, India. All other chemicals and solvents used were of analytical reagent grade and procured locally.

Experimental protocols were reviewed and approved by the institutional Animal Ethics Committee and conform to the Indian National Sciences Academy guidelines for the use and care of experimental animals in research (Animal House Registration No 25/1999 CPCSEA).

**Animal models**

**Acute inflammation:**

Carrageenin induced hind paw edema in rats—

Edema was produced acutely by sc injection (0.1ml) of carrageenin (1%w/v) into the plantar region of the hind paws of the rat according to the method of Winter et al.6.

**Sub-chronic inflammation:**

Cotton pellet granuloma in rats—The cotton pellet granuloma was produced in rats as per Winter and Porter7 with slight modification. Pellets, weighing exactly 10 mg each were made by rolling between fingers. The cotton pellets were sterilized in an autoclave for 30 min at 120°C under 15lb pressure. Four pellets were inserted subcutaneously in the ventral region, two on either side, in each rat under light ether anaesthesia.

**Chronic inflammation:**

Freund’s adjuvant induced arthritis—Freund’s adjuvant (0.1ml, complete) was injected sc into the plantar region of the right hind paw of the rat by the method of Newbould et al.8.

**Analytical procedure**

**Polyamines determination** —Polyamines contents were assayed by the method of Seiler and Askar9 and Seiler and Weichmann10 with slight modification. The method involves homogenization of tissue with ice-cold 0.2N perchloric acid and after centrifugation for 15 min at 800 g the supernatant was separated into test tubes. This supernatant was then treated with 1 ml of dansyl chloride (10 mg/2.5 ml of acetone) and saturated with sodium carbonate. This reaction mixture was kept at room temperature overnight in dark to allow dansylation of polyamines. To this, 10 mg proline dissolved in 0.2ml water was added in order to react with the excessive dansyl chloride. After 2 hr, the dansylated polyamines were extracted from the reaction mixture with 2×10 ml (two times with 10 ml) of benzene. The organic layer was taken to dryness in a stream of nitrogen at 40°C and the residues were reconstituted in 0.1ml of toluene:ethyl acetate (7:3) mixture. The reconstituted dansylated amine mixture was quantitatively applied onto a 20×20 cm thin layer plate (silica gel G) using Linomat 5 sample applicator. The standards putrescine, spermidine and spermine in different concentrations after dansylation were co-chromatographed with samples for the identification of respective spots and for the preparation of standard graph. The plates were developed in a TLC chamber saturated with a mobile phase system consisting of Chloroform:Toluene:Triethylamine (60:28:12). The Rf values for putrescine, spermidine and spermine were 0.17, 0.33 and 0.45 respectively. The thin layer plates were dried for 10 min at room temperature and the CAMAG scanner at 366 nm scanned the plates. The peak area of sample was extrapolated on a standard graph for calculating the concentration of the respective polyamine.

**DNA estimation** — DNA was isolated from the inflamed tissue as per Plummer11 and was estimated spectrophotometrically by the diphenylamine reaction11.

**Protein estimation** — Approximately 100 mg tissue was homogenized in 2 ml saline. A 0.1 ml of this homogenate was used for protein analysis by the method of Lowry et al12.

Levels of polyamines in different types of inflammation were determined as follows:

Carrageenin induced hind paw edema—The rats were sacrificed under light ether anaesthesia at different time intervals i.e., 3, 6, 12 and 24 hr after carrageenin injection, polyamines, DNA and proteins were determined in edematous tissue by removing edema tissue along with exudates from the whole paw excluding the cartilage and nerves.

Cotton pellet granuloma test —On the 8th day after subcutaneous implantation of cotton pellets, the animals were sacrificed under light ether anaesthesia and cotton pellets were removed and granulomatous tissue formed around the pellet was separated for polyamines, DNA and protein assays.

In case of Freund’s adjuvant induced arthritis, on the 22nd day after Freund’s adjuvant injection, rats were sacrificed under light ether anaesthesia and polyamines, DNA and proteins were assayed in edematous tissue by removing from paw edema excluding the cartilage and nerves.
Effect of different anti-inflammatory drugs on polyamine levels in inflamed tissue—A steroidal drug, dexamethasone (5 mg/kg), a non-specific cyclooxygenase inhibitor, diclofenac sodium (10 mg/kg) and a selective cyclooxygenase 2-inhibitor, valdecoxib (10 mg/kg) were used in animal models of inflammation. These drugs were selected because they induce anti-inflammatory activity through different mechanism(s).

In case of carrageenin hind paw edema, drugs were administered orally 1 hr prior to carrageenin injection and animals were sacrificed under light ether anaesthesia 6 hr after carrageenin injection and edematous tissue was removed for various estimations. The 6 hr interval was chosen for polyamines determination as maximum inflammation was observed at 6 hr following carrageenin injection and also the anti-inflammatory drugs shown/reported to have half-life approximately 6-8 hr.

In case of cotton pellet granuloma, drugs were administered orally daily for 7 days and on the 8th day animals were sacrificed under light ether anaesthesia. The granulomatous tissue was removed carefully from around the pellets for determining polyamines, DNA and protein as described earlier.

Statistical analysis—One-way ANOVA with Dunnett's post test was performed using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, (www.graphpad.com).

Results

In acute inflammation (carrageenin paw edema), there was an insignificant decrease in polyamines upto 12 hr after carrageenin injection. However, there was a significant increase in all the three polyamines, putrescine, spermidine and spermine in edema tissue at 24 hr. Similarly, in chronic inflammatory model (adjuvant induced arthritis), there was a significant increase in polyamines content in the edematous tissue. In granulomatous tissue of sub-chronic inflammatory model (cotton pellet granuloma), there was a significant increase in polyamines levels as compared to the content of the normal connective tissue. The protein and DNA content of the inflamed tissue were significantly increased as compared to the normal tissue (Table 1).

In carrageenin edema, treatment with dexamethasone induced an increase in levels specifically of putrescine whereas increase in spermidine and spermine remained insignificant. Diclofenac treatment elicited a significant decrease in the levels of putrescine and spermine while valdecoxib significantly decreased spermine content of edematous tissue (Table 2). Dexamethasone treated cotton pellet granuloma rats also showed an increase in putrescine similar to that observed in carrageenin rat paw edema test. However, diclofenac and valdecoxib treated rats showed significant decrease in putrescine level in cotton pellet granuloma as compared to untreated control. The protein and DNA content of the inflamed tissue were significantly decreased with anti-inflammatory drug treatment as compared to the normal tissue (Tables 2 and 3).

The present results show that polyamines levels, and protein and DNA content were increased in edematous and granulomatous tissues. Treatment with steroidal drug, dexamethasone elevated polyamine putrescine but inhibited both protein and DNA content of inflamed tissue. The non-steroidal drugs, diclofenac and valdecoxib decreased polyamines along with decrease in the DNA and protein contents of the inflamed tissue.

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<table>
<thead>
<tr>
<th>Edema type and time interval (hr)</th>
<th>Edema</th>
<th>Putrescine (mmol/g of tissue)</th>
<th>Spermidine (mmol/g of tissue)</th>
<th>Spermine (mmol/g of tissue)</th>
<th>Protein (mg/g of tissue)</th>
<th>DNA (mg/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>----</td>
<td>5.13±0.4000</td>
<td>0.046±0.004</td>
<td>0.272±0.012</td>
<td>49.5±1.50</td>
<td>1.19±0.01</td>
</tr>
<tr>
<td>Carrageenin (3)</td>
<td>7.35±1.0</td>
<td>5.835±1.255</td>
<td>0.045±0.003</td>
<td>0.220±0.009</td>
<td>89.25±3.95**</td>
<td>1.81±0.07**</td>
</tr>
<tr>
<td>Carrageenin (6)</td>
<td>9.3±1.0</td>
<td>4.080±0.221</td>
<td>0.038±0.003</td>
<td>0.294±0.013</td>
<td>82.25±4.37**</td>
<td>2.2±0.14**</td>
</tr>
<tr>
<td>Carrageenin (12)</td>
<td>6.3±0.4</td>
<td>5.060±0.512</td>
<td>0.064±0.002</td>
<td>0.283±0.009</td>
<td>71.75±2.78**</td>
<td>2.55±0.13**</td>
</tr>
<tr>
<td>Carrageenin (24)</td>
<td>1.25±0.3</td>
<td>8.92±1.067**</td>
<td>0.092±0.007**</td>
<td>0.347±0.021**</td>
<td>77.00±2.48**</td>
<td>2.17±0.12**</td>
</tr>
<tr>
<td>Granuloma tissue</td>
<td>----</td>
<td>25.96±0.920</td>
<td>0.089±0.013</td>
<td>0.358±0.086</td>
<td>94.5±4.94</td>
<td>1.69±0.13</td>
</tr>
<tr>
<td>Arthritic rat paw</td>
<td>10.8±2.0</td>
<td>20.16±0.600**</td>
<td>0.208±0.014**</td>
<td>0.412±0.018**</td>
<td>85.33±2.03**</td>
<td>2.72±0.086**</td>
</tr>
</tbody>
</table>

P values: ** <0.01 as compared to normal (one-way ANOVA)

Edema in terms of manometer readings (manometers 100 divisions correspond to 13.6g of mercury)
Discussion

In the present experiments, the polyamines levels were elevated in edematous and granulomatous tissues in acute, sub-chronic and chronic inflammatory animal models. However it was observed in acute model, that the increase in polyamines occurs at later stage only after the peak edema formation. Hence it is likely that the increase in polyamines during inflammation may be due to induction of ornithine decarboxylase, a rate-limiting enzyme in polyamines biosynthesis in liver\textsuperscript{13,14} and other tissues including connective tissue especially in chronic inflammation. The increase may also be partly due to local release of polyamines from the injured cells\textsuperscript{15}. The elevated polyamines observed during inflammation will trigger negative immune regulators by its action on lymphocytes\textsuperscript{16}, neutrophils locomotion\textsuperscript{17} and natural killer cell activity\textsuperscript{18}. Some of the polyamines especially spermine has been implicated in macrophage cytokine synthesis inhibition\textsuperscript{19,20}. All the aforementioned cellular events clearly indicate that polyamines are acting as endogenous anti-inflammatory substances. Alternatively it can be presumed that increased polyamines might act as endogenous regulators of the inflammatory process.

Polyamines play crucial role in DNA, protein synthesis and are thus involved in cell proliferation. There was a significant elevation in DNA and protein content of edematous and granulomatous tissue with the concomitant increase in polyamines suggesting direct involvement of polyamines in the repair process during inflammation. However, such a relationship is difficult to extend in carrageenin edema where infiltration of fluid and cell migration from plasma plays a major role. The simultaneous increase of polyamines with DNA and protein in the inflamed tissue suggest active participation of polyamines in the healing process. They may be involved either in the regeneration of new tissue in place of the inflamed tissue as evident from the acute inflammation or they may be involved in the formation of fibrous tissue as seen in the chronic inflammation.

Dexamethasone treatment in carrageenin edema as well as in cotton pellet granuloma rats elevated levels of putrescine, suggesting steroidal drugs positively modulates polyamines at the site of inflammation. However, protein and DNA contents decreased with dexamethasone largely due to decreased proliferation in the inflamed tissue. This is in agreement with the earlier findings that dexamethasone activity is mediated through putrescine synthesis\textsuperscript{5}. Furthermore, dexamethasone is also known to stimulate ornithine decarboxylase activity in rat liver\textsuperscript{20} as well as in connective tissue which in turn induce vasoregulin, an endogenous anti-inflammatory protein. Hence, it can

<table>
<thead>
<tr>
<th>Drug treatment (mg/kg, po)</th>
<th>Edema (mmol/g tissue)</th>
<th>Putrescine (nmol/g tissue)</th>
<th>Spermidine (mmol/g tissue)</th>
<th>Spermine (mmol/g tissue)</th>
<th>Protein (mg/g tissue)</th>
<th>DNA (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>10.3±0.6</td>
<td>3.99±0.51</td>
<td>0.04±0.004</td>
<td>0.30±0.016</td>
<td>81.5±6.40</td>
<td>2.28±0.13</td>
</tr>
<tr>
<td>Dexamethasone (5)</td>
<td>0.6±0.01</td>
<td>12.6±1.25**</td>
<td>0.04±0.002</td>
<td>0.30±0.015</td>
<td>52.00±4.55**</td>
<td>1.48±0.099**</td>
</tr>
<tr>
<td>Valdecoxib (10)</td>
<td>5.6±0.2</td>
<td>3.95±1.048</td>
<td>0.03±0.005</td>
<td>0.22±0.009**</td>
<td>59.5±6.55**</td>
<td>1.77±0.056**</td>
</tr>
<tr>
<td>Diclofenac (10)</td>
<td>2.6±0.2</td>
<td>0.86±0.104*</td>
<td>0.02±0.001</td>
<td>0.21±0.012**</td>
<td>57.25±3.20*</td>
<td>1.19±0.034**</td>
</tr>
</tbody>
</table>

\(P\) values: \(*<0.05; \quad **<0.01\) as compared to normal (one-way ANOVA).

Table 2 — Levels of polyamines in the edematous rat paw tissue following anti-inflammatory drugs treatment in the carrageenin edema

| Values are mean \(\pm\) SE from 5 observations in a group |

Table 3 — Levels of polyamines in the granulomatous tissue separated from around the pellets in the Cotton pellet granuloma

| Values are mean \(\pm\) SE from 5 observations in a group |
be presumed that polyamines may act as second mediators of glucocorticoids. However, this needs to be confirmed by further experiments.

In the present findings, diclofenac and valdecoxib significantly lowered the levels of polyamines suggesting non-steroidal drugs negatively modulate polyamines at the site of inflammation possibly by the induction of SSAT (sperridine/sperrmine acetyltransferase) as well as inhibition of ODC (ornithine decarboxylase). It has also been reported that NSAIDs affect polyamines by inducing SSAT (spermidine/spermine acetyltransferase) as well as inhibition of ODC (ornithine decarboxylase). The induction of SSAT (spermidine/spermine acetyltransferase) as well as inhibition of ODC (ornithine decarboxylase) has also been reported. The proliferation at the site of inflammation is hindered which is reflected in decreased DNA and protein contents. It is clear from the present results that non-steroidal drugs hinder natural healing process by inhibiting cell proliferation.

In conclusion, polyamines levels are elevated at the site of inflammation indicating their active involvement in proliferation. Steroidal drugs positively modulated polyamines levels whereas non-steroidal drugs negatively modulated their levels in inflammation or inflammatory process.

References