Sheep uterine tissue is rich in the enzyme 5-lipoxygenase (5-LOX) that generates 5-hydroperoxyeicosatetraenoic acid (5-HPETE) on incubation with arachidonic acid (AA). The present study focuses on the ability of the enzyme 5-LOX to further act as 5, 6 leukotriene A4 (LTA4) synthase using 5-HPETE as a substrate. On incubation of 5-LOX with 5-HPETE and analysis of products on reversed-phase high-performance liquid chromatography, a prominent peak with a characteristic conjugated triene peak having absorption maxima of 261, 271, and 281 nm appeared. Based on cochromatography, the peak was identified as 5(S), 6(S)-dihydroxyeicosatetraenoic acid (5[S], 6[S]-diHETE), the non-enzymatic product of 5, 6 LTA4. As the synthesis of 5,6-LTA4, from 5-HPETE requires 8-LOX activity, the present study demonstrates the dual LOX, 5- and 8-LOX activity for uterine 5-LOX, that is, 5-,6- LTA4 synthase in sheep uterus. In addition, endogenous 5,6-LTC4, the glutathione conjugate of 5,6-LTA4, was also observed in the sheep uterine homogenate, further demonstrating the synthesis of 5,6-LTA4 in the sheep uterine tissue.

1. INTRODUCTION

Fatty acids like arachidonic acid (AA) undergo oxygenation to their hydroperoxy derivatives by enzymes lipoxygenases (LOX) [1]. The bioactive compounds such as leukotrienes (LT), lipoxins, and others are generated from the primary products of the LOX pathway [2,3]. The LTs consist of LTβ, which is pro-inflammatory and the cysteinyl-LTs C4, D4, and E4 which are spasmodic [4]. The LTs are formed by the oxygenation of AA to hydroperoxy eicosatetraenoic acid (HPETE), which, in turn, gets converted to LTA4. LTA4 gets converted to LTB4 by selective hydrolysis or to LTC4 by conjugation with reduced glutathione (GSH). These LTs perform different functions in various organs and cells.

The cysteinyl LTs (CysLTs) interact through G-protein coupled specific cell surface receptors CysLT1 and CysLT2, which mediate the responses during inflammation reactions [5-7]. In general, the CysLTs have potency in smooth muscle contraction higher than that of histamine and have shown potent muscle contractions [8]. LTC4 and D4 exhibit greater contractile action in human bronchial or parenchymal strips, compared to prostaglandin F2α or histamine, and are the major mediators of asthma [9]. The LT receptor antagonists, montelukast and zafirlukast, are in fact are the anti-asthmatic drugs in the market. LTβ4 stimulates innate immune response being a major bioactive peptide-free LT and involves in various immunological functions including chemotaxis, adherence of neutrophils, and increased vascular permeability [10-12]. LTβ4 activates inflammatory cells by binding to surface receptors of cells of type BLT1 and BLT2 and activates G-protein coupled intracellular signaling cascades [13].

There are a number of studies showing the involvement of 5-LOX and LTs in uterine function and during labor. During labor, the 5-LOX gene gets expressed higher in human choriodecidua [14], levels of 5-LOX products are increased in the amniotic fluid [15], and myometrial contractility was stimulated by LTs [16]. LOX gene expression was shown to vary in baboon intrauterine tissues at different stages of late pregnancy and parturition [17]. In cattle, during the estrous cycle and early pregnancy, LTs were shown to influence the secretion of progesterone and prostaglandins [18] and a recent report indicates that 12-LO and 5-LO pathways complement the production of LTs in the bovine uterus [19]. All the reported studies strongly indicate the importance of 5-LOX as well LTs in uterine function and metabolism.

The mechanism of action of 5-LOX involves stereospecific abstraction of hydrogen at C-7 of AA to yield 5-HPETE. In the following reaction, 5-HPETE is known to get converted to LTA4 (LTA4, 5S-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid), which is highly unstable. There are reports to demonstrate that a single enzyme possesses dual LOX activities of 5- and 8-LOX and thereby 5-LOX and LTA4 synthase activities [20-22]. Even though there are several studies indicating the presence of LOX enzymes and the existence of LTs in the uterine tissues, comprehensive studies are lacking in...
connecting LOX enzymes and LT in the uterus. In this study, we provide evidence that a single enzyme 5-LOX from sheep uterus metabolizes AA to 5-HPETE and further to 5,6-LTA₄ by its 5-, 8-dual LOX activity. Apart from having 5,6-LTA₄ synthesis in the sheep uterus, the present work also provides evidence for the existence of 5,6-LTC₄ in the sheep uterus, a product formed from 5,6-LTA₄ by the conjugation of GSH. This finding is in addition to the formation of 14, 15-LTC₄ from 14, 15-LTA₄ by the action of its synthase activity as reported by the earlier studies [23,24].

2. MATERIALS AND METHODS

2.1. Chemicals

Phenyl methyl sulfonyl fluoride (PMSF) was procured from Sigma Chemicals Co., St. Louis, USA, standard 5(S),6(S) diHETEs, 5(S),6(R) diHETEs, and 5,6-LTC₄ are from Cayman Chemicals, Ann Arbor, USA. AA was from Nu Chek Prep, USA. Polyclonal rabbit anti-5-LOX antibodies were from Thermo Fisher Scientific, USA, DE-52 was from Whatman. All other chemicals were purchased from Qualigens, India, and are of analytical grade.

2.2. Tissue Collection

Mature and non-pregnant sheep uterine tissue was collected from a local slaughterhouse, followed by removal of ovaries, cervix, and fat tissue and immediately used in the experiments.

2.3. Extraction of Sheep Uterine 5-LOX

The extraction procedure was followed as described earlier [25]. A 20% homogenate was prepared from the non-pregnant sheep uterine tissue in 150 mM potassium phosphate, pH 7.4 buffer containing 2 mM MgCl₂, 1 mM KCl, 1 mM ascorbic acid, 2 mM CaCl₂, 10 mM sodium metabisulfite, 1 mM PMSF and 250 mM sucrose. The homogenate was filtered through a cheese cloth and subjected to centrifugation at 12,000 rpm for 20 min. The supernatant was further subjected to high-speed centrifugation at 33,000 rpm for 1 h and the resulting cytosol was loaded onto DE-52 in a batch-wise fashion. Since flow-through contained most of the activity, washing of DE-52 with homogenization buffer and elution with 0.5 M NaCl was discontinued in the later extraction procedures. Precipitation of proteins in the flow-through was achieved by ammonium sulfate (NH₄)₂SO₄ fractionation at 50–70% saturation and the fractionated protein was assayed for enzyme activity. Protein concentration was estimated by the method of Lowry [26].

2.4. Immunoblot of 5-LOX

100 µg protein of 50–70% ammonium sulfate fraction of sheep uterine cytosol was separated on a 10% SDS-PAGE by the method of Laemmli [27]. The proteins were subjected to Western blot analysis by the method of Towbin et al. [28]. The polyclonal 5-LOX specific antibodies were used to bind to the fractionated proteins and blots were developed with goat anti-rabbit IgG-horseradish peroxidase.

2.5. LOX Enzymatic Activity

Enzyme activity was measured by the spectrophotometric method developed by Ben-Aziz et al. [29]. Sheep uterine 5-LOX (30–100 µg) was dissolved in 150 mM citrate phosphate buffer, pH 5.5 and the reaction was followed after the addition of 250 µM AA for 1 min at 235 nm. The enzyme activity was expressed as µmoles of hydroperoxides formed per min per mg protein.

2.6. 5-LOX Products of AA

The extracted 5-LOX enzyme was used to generate products by incubating with AA (250 µM) in 150 mM citrate phosphate buffer and pH 5.5 as 100 ml reactions. The incubation time was 2 min and after that, the reaction mixture was acidified to pH 3.0 using 6N HCl. The generated 5-HPETEs were extracted into equal volumes of hexane: ether mixture. The HPETEs in the organic phase were dried completely and the residue was dissolved in ethanol or solvent of hexane: propane-2-ol: acetic acid in 1000:15:1 ratio.

The 5-LOX products were initially analyzed by thin layer chromatography (TLC) using a solvent system of diethyl ether: hexane:acetic acid (60:40:1) further subjected to straight-phase HPLC using CLC-SIL (25 × 0.4 cm) column (Shimadzu model equipped with SPD 6AV detector and C4A chromatopac). The mobile phase used was hexane: propane-2-ol: acetic acid in 1000: 15:1 ratio and the column flow rate was adjusted to 1 ml/min. The products from the column eluent were monitored at 235 nm and spectral analysis was performed for visible peaks. The identification of 5-HPETE from the peaks was confirmed by cochromatography with the standard. The standard 5-HPETE was generated in the laboratory using potato 5-LOX by the method of Reddanna et al. [30].

2.7. 5,6-LTA₄ Synthase (LTA₄) Activity

Assay of LT synthase activity and extraction of products were followed as described by Sailesh et al. [23]. Active sheep uterine 5-LOX enzyme (100 µg) was incubated with 150 µM of 5-HPETE in 150 mM citrate-phosphate buffer, pH 5.5 for 10 min in a total reaction mixture of 10 ml. Solvent system of hexane: ether (1:1) was used to extract the products and is evaporated under nitrogen. The residue was dissolved in methanol and TLC analysis was performed with a mobile phase of diethyl ether: hexane: acetic acid (60:40:1). Otherwise, the residue was dissolved in reversed-phase high-performance liquid chromatography (RP-HPLC) solvent system and on RP-HPLC using Waters C-18 column (25 × 0.4 cm). The solvent employed for RP-HPLC was methanol: water:acetic acid (65:35:0.1) adjusted to pH 6.8 with ammonium hydroxide. The flow rate of the column was adjusted to 1 ml/min and the products were monitored at 268 nm for 30 min. The individual peaks were further subjected to spectral analysis by ultraviolet (UV)-visible spectrophotometer.

### Figure 1:

Western blot analysis of sheep uterine 5-lipoxygenase (5-LOX). Lane 1 and 2, 5-LOX was separated on 10% SDS-PAGE and cross-reacted with commercial antibodies against 5-LOX [Lane 3, pre-stained protein markers]
The 5,6-LTA₄ non-enzymatic hydrolyzed products, 5(S),6(S)-diHETEs, and 5(S), 6(R)-diHETEs were identified based on the triene spectra and cochromatography with standards.

2.8. Extraction of Endogenous LTs

The sheep uterine endogenous LTs were extracted based on the procedure described by Huwyler and Gut [31]. The extraction procedure involved mixing sheep uterine cytosol with iso-propanol in three volumes and the addition of 5N formic acid to acidify the reaction mixture to pH 3.0. Stirring was followed for 30 min after the addition of three volumes of methylene chloride solution. The reaction components were centrifuged at 10,000 × g for 10 min to separate the aqueous and organic phases. To the organic phase, 100 µl of distilled water was added after careful removal of the aqueous and interphase layers. After recentrifugation of the sample, the supernatant was concentrated to 50 µl and the compounds in the sample were analyzed on reverse phase HPLC using a C-18 CLC-ODS column (25 × 0.46 cm) with a flow rate of 1 ml/min. The eluent used was methanol: water:acetic acid that was used in the ratio of 72:28:0.1, with 50 µM EDTA and pH 6.0. The effluent was monitored for peaks at an absorbance of 280 nm and the prominent peaks were further subjected to UV spectral analysis.

Figure 3: (a) Reverse-phase high-performance liquid chromatography (RP-HPLC) of standard 5(S), 6(S) dihydroxy eicosatetraenoic acid (5[S], 6[S]-diHETE), (a.1) Spectral analysis of peak with RT 35.46 (b) RP-HPLC analysis of 5-hydroperoxy eicosatetraenoic acid product profile with uterine 5-lipoxygenase: Assay of 5,6 leukotriene A₄ synthase activity, (b.1) Spectral analysis of peak with RT 35.63
3. RESULTS

3.1. Isolation and Western Blot Analysis of 5-LOX
High activity of the 5-LOX was found in 50-70% (NH₄)₂SO₄ precipitate of the DE-52 flow-through fraction. The identity of the enzyme was also confirmed by Western blot analysis using 5-LOX-specific antibodies. The molecular mass of the protein was found near 66 kDa [Figure 1].

3.2. LTA₄ Synthase Activity of 5-LOX
The role of sheep uterine 5-LOX in the biosynthesis of LTs was analyzed by reacting the enzyme with sheep uterine 5-LOX-generated 5-HPETE. The 5-HPETE of sheep 5-LOX generated [Figure 2, Lane 3 and 4] was compared with the standard 5-HPETE [Figure 2, Lane 2]. The extraction of LT products was done in a hexane: ether (1:1) solvent system and further analyzed on TLC and HPLC. The amount of 5-HPETE after incubation with 5-LOX was drastically decreased on TLC, confirming that it might have been used up as the substrate for the formation of LTs and further to diHETEs [Figure 2, Lane 5]. The diHETEs were unable to be detected on TLC, though different solvent systems were employed.

HPLC monitoring of the column eluent at 268 nm, prominent peak with RT 35.63 min was observed [Figure 3b], which coeluted with the standard 5(S),6(S)-diHETE. The peak has shown the characteristic 5(S),6(S)-diHETE spectrum with absorption maxima of 261, 271, and 281 nm [Figure 3b.1]. A standard 5(S),6(S)-diHETE, when run separately showed a peak with RT of 35.46 min and characteristic spectra [Figure 3a and a.1], further confirming the formation of diHETEs in the sample.

3.3. Extraction of Endogenous LTs
From the sheep uterine homogenate, the LTC₄ peptideo LTs are extracted and analyzed on RP-HPLC where the products have shown a peak with a retention time of 12.2 min [Figure 4b]. A characteristic conjugated triene spectrum with absorption maxima at 270, 280, and 290 nm was observed for the peak fraction [Figure 4b.1]. The retention time of this product coincided with standard 5,6 LTC₄ [Figure 4a and 4a.1] and both of them coeluted as a single homogeneous peak having RT of 11.9 min.

4. DISCUSSION
The structural and inflammatory cells’ outer plasma membrane contains a specific rhodopsin class of heptahelical receptors and LTs act by binding to them [32,33]. Increased levels of intracellular
calcium and decreased levels of intracellular cyclic AMP are the effects of LTs through cytoplasmic G proteins. The downstream kinase cascades get in turn activated and result in the alteration of various cellular activities which include the basic act of motility to higher-order transcriptional activation. Alternatively, LTA4 gets hydrolyzed non-enzymatically into 5,6-di HETE (having absorption maxima at 272 nm) and 5,12-di-HETEs (having absorption maxima at 268.5 nm).

Our earlier studies in the sheep uterus under in vitro conditions have shown the presence of 12- and 15- dual LOX as well as an abundant presence of 5-LOX [23,25]. The identity of 5-LOX was confirmed by immunoblot analysis and the molecular weight of the protein was found at near 66 kDa, which could be a slightly degraded compound of original protein and it was reported as 72 kDa in porcine leukocytes [34] and 80 kDa in human leukocytes [35].

Formation of 5, 6-LTs in the sheep uterine system was not reported so far, though the presence of 14, 15-series of LTs was mentioned earlier [23]. When the sheep uterine 5-LOX was incubated with 5-HPETE, the formation of 5(S), 6(S)-di HETEs with their characteristic spectra with maximum absorption at 271 nm suggests the formation of 5,6-LTA4 and its non-enzymatic hydrolysis to diHETEs. The formation of 5,6-diHETEs from 5-HPETE in the presence of 5-LOX attributes to the 5- and 8-dual LOX activity for uterine 5-LOX. Similar reports of LTA4 synthase activity of 5-LOX were mentioned earlier in other species [20-23] and these reports strongly support our understanding of the ability of the 5-LOX as dual LOX.

Another finding in the present study was the presence of endogenous 5,6-LTC4, which strongly provides information for the formation of total series of 5, 6-LTs in the sheep uterus. Our findings are further augmented by the studies on purification and characterization of LTC4 synthase from the sheep uterus by earlier studies [24]. The preference of the substrate 14, 15-LTA4 over 5, 6-LTA4 by LTC4 synthase isolated from sheep uterus [24] indicates the possibility of the existence of another 5,6-LTA4 specific LTC4 synthase, which needs further investigation. The LTs formed in the sheep uterus probably play a very important roles in reproductive functions as evidenced by the earlier studies showing elevated mRNA expression of 5-LOX and LTB4 (receptor for LTB4) and CysLTR2 (receptor for LTC4) [19]. It is well established that LTB4 is luteotrophic and LTC4 is luteolytic, regulating the formation of progesterone and thus maintenance of pregnancy [36,37]. In addition, LTs are known to regulate the production of prostaglandins, which play a key role in the regulation of parturition [38] and thus in the physiology of the uterus.

5. CONCLUSION

Based on the finding of this study, a model has been proposed for the metabolism of AA by 5-LOX in the sheep uterus [Figure 5] AA gets converted into 5-HPETE by 5-LOX and 5-HPETE in turn majorly gets converted into 5,6-LTA4 by the dual action of 5-LOX. The 5,6-LTA4 so formed gets modified into LTB4 or into LTC4 by the hydrolase or synthase activities, respectively. As 5,6-LTA4 is highly unstable, if it has not got transformed into potential LTs, it may undergo non-enzymatic hydrolysis into 5,6- or 5,12-diHETEs (mostly under in vitro conditions). There are other fates for 5-HPETE as getting converted enzymatically into 5, 12-diHETE by 12-LOX or into 5,15-diHETE by 15-LOX activities.

6. AUTHORS’ CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the present journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

Financial support from University Grants Commission, Govt. of India, New Delhi through post-doctoral research award to ASP is thankfully acknowledged. Infra structural facilities provided by BVB management to ASP are highly acknowledged. Senior Research Associate fellowship for KAK (CSIR-SRA (13(8956-A)/2017 Pool from CSIR is highly acknowledged.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

Ethical approval is not applicable as the study was conducted on the remains of the already slaughtered animal.

10. DATA AVAILABILITY

All data generated through experiments is included within this research article.
