Liver X receptors and epididymal epithelium physiology

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Abstract

Aim: To investigate the roles of liver X receptors (LXR) in the lipid composition and gene expression regulation in the murine caput epididymidis. LXR are nuclear receptors for oxysterols, molecules derived from cholesterol metabolism that are present in mammals as two isoforms: LXRα, which is more specifically expressed in lipid-metabolising tissues, such as liver, adipose and steroidogenic tissues, and macrophages, whereas LXRβ is ubiquitous. Their importance in reproductive physiology has been sustained by the fact that male mice in which the function of both LXR has been disrupted have fertility disturbances starting at the age of 5 months, leading to complete sterility by the age of 9 months. These defects are associated with epididymal epithelial degeneration in caput segments one and two, and with a sperm midpiece fragility, leading to the presence of isolated sperm heads and flagella when luminal contents are recovered from the cauda epididymidis.

Methods: The lipid composition of the caput epididymidis of wild-type and LXR-deficient mice was assessed using oil red O staining on tissue cryosections and lipid extraction followed by high performance liquid chromatography or gas chromatography. Gene expression was checked by quantitative real time polymerase chain reaction.

Results: Using LXR-deficient mice, we showed an alteration of the lipid composition of the caput epididymidis as well as a significantly decreased expression of the genes encoding SREBP1c, SCD1 and SCD2, involved in fatty acid metabolism.

Conclusion: Altogether, these results show that LXR are important regulators of epididymal function, and play a critical role in the lipid maturation processes occurring during sperm epididymal maturation. (Asian J Androl 2007 July; 9: 574–582)

Keywords: epididymis; liver X receptors; nuclear receptors; lipids; cholesterol; gene expression

1 Introduction

Liver X receptors (LXR) are members of the nuclear receptor superfamily, and are bound and activated by a specific class of oxysterols derived from endogenous cellular cholesterol metabolism [1]. These receptors possess transcription factor properties, working as obligate heterodimers with retinoid X receptors [2]. The LXRα isoform is mainly expressed in tissues with active lipid metabolism, whereas LXRβ is ubiquitously represented. The physiological functions of LXR have been extensively studied in the past decade using knock-out animals for each isoform. They appear to be mainly involved in lipid metabolism in the control of both fatty acid and cholesterol homeostasis (reviewed by [3]).

Among the various physiological functions identified so far, LXR have been shown to be important for reproductive function at different levels, because mice invalidated for both isoforms (lxra;β−/−) have difficulties in procreating after the age of 7 months. Interestingly, this is specific for the double-deficiency. Mice deficient in only one of the two isoforms are still fertile after this age. Investigations [4] have shown that LXRα-deficient mice have lower levels of testicular testosterone, which
correlates with a higher apoptotic rate of germ cells. LXRβ-deficient mice showed increased lipid accumulation in Sertoli cells and a lower proliferation rate of germ cells [4]. In lxrα;β-/- mice, fatty acid metabolism was affected, and the retinoid acid signaling pathway also altered. The combination of these alterations might explain the deleterious phenotype of infertility observed only in lxrα;β-/- mice [4]. Besides this testicular phenotype, we previously showed that spermatozoa obtained from the epididymis have a midpiece fragility, identified by an increased number of broken cells appearing as either a separate flagellum or head [5]. The epididymal phenotype was characterized by an altered epithelium, specifically located in segments one and two of the caput epididymidis, which presented cells with a reduced height, and an undetermined material present in the lumen. It appeared that this phenotype was not secondary to the decreased testosterone level, as it was not reversed by daily injections of testosterone for 4 months.

Because the molecular cause of the epithelial disruption of the epididymis was unclear, we investigated the consequences of LXR deficiency (lxrα-/-, lxrβ-/- and lxrα;β-/-) on lipid composition and on some lipid-related gene expression in the caput epididymidis to determine which isoform could be responsible for the regulation of lipid homeostasis in this organ.

2 Materials and methods

2.1 Animals

The generation of LXR-deficient mice has been described elsewhere [6–7]. Male mice of the BL6 × 129 Svj hybrid strain were reared in a temperature-controlled (22°C) atmosphere with a 12 h:12 h Light: Dark cycle. Mice were handled according to the Guidelines on the Use of Living Animals in Scientific Investigations, which were approved by the Regional Ethic Committee (authorization CE2-04). Tissues were dissected, as described earlier [5], frozen in liquid nitrogen and stored at −80°C before use. For cryosections, tissues were included in Optimal Cutting Temperature (Electron Microscopy Sciences, Hatfield, PL, USA) and frozen under liquid nitrogen vapour.

2.2 Oil red O staining

Lipid staining of each organ collected was performed on 8-µm-thick cryosections with 1,2 propanediol (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 4 min, as previously described [5].

2.3 High performance thin layer chromatography (HPTLC)

Epididymal lipids were extracted by the Folch method, with modifications [8]. Three different tissue samples (caput epididymidis) from three different animals were analyzed for each genotype. Cholesterol, cholesteryl esters and phospholipids were separated by high-performance thin-layer chromatography (HPTLC; 10 × 10 cm; Merck, Lyon, France) with methyl acetate, propan-2-ol, chloroform, methanol and 0.25% (w/v) aqueous KCl (25:25:25:10:9, v/v) [8]. Lipid-containing regions of the chromatogram were visualized by treatment with a 10% (w/v) CuSO4 and 8% (v/v) H3PO4 solution and heated at 180°C. The chromatograms were scanned, and spots were quantified via densitometry (Quantity One; Bio-Rad, Marnes-la-Coquette, France) by reference to different concentrations of standards on each HPTLC migration. Standard dosage values gave curves with linear-regression coefficients (R²) of 0.90 or greater.

2.4 Gas chromatography

Total lipids were extracted by a classical chloroform/methanol method based on the Folch method, and the final lipid extract was diluted in a volume of 100 µL chloroform. Total lipids were then fractionated in non-phosphorylated lipids, neutral lipids (NL) and phospholipids (PL) using a Sep-pak column (Waters, Guyancourt, France). Briefly, after washing the column with 4 mL chloroform, total lipids were adsorbed on the column, the NL were eluted with 4 mL chloroform and the PL by an elution step using 8 mL methanol. Lipids were then evaporated under nitrogen and diluted in 200 µL toluene. Lipids were then methylated for 20 min at 20°C under nitrogen with 100 µL of 2 mol/L sodium methanolate (Sigma, Saint-Quentin Fallavier, France), followed by a 20 min incubation with 500 µL 14% BF3/methanol solution and heated at 180°C. The methyl esters obtained were concentrated by evaporation under nitrogen, diluted in 200 µL hexane and stored at −80°C until further use.

Before analysis, samples were filtrated on a Florisil column, eluted with a mixture of hexane: ethyl ether (95:5, v:v), evaporated and diluted in a known volume of hexane. The analysis was performed on a GC trace gas chromatograph (Thermo Electron, Courtaboeuf, France) equipped with a capillary DBWAX column (30 m, 0.25 mm, 0.25 µm thick, JW Scientific, Folsom, CA, USA). The injector was a Split-Splitless type and the detector a flame-ionisation detection type. The fatty acid methyl esters were characterized in quality and quantity by comparing
their retention times with those obtained from a known mixture (MIX37 from Sigma).

2.5 Quantitative reverse transcriptase polymerase chain reaction
Total RNA was isolated using the Trizol method (Invitrogen, Cergy Pontoise, France) according to the manufacturer’s instructions. cDNA was synthesized with Imprint II Reverse Transcriptase (Promega, Charbonnières, France) and random hexamer primers (Promega, Charbonnières, France) according to the manufacturer’s recommendations. Real-time polymerase chain reaction was performed on an iCycler (Biorad, Marnes-la-coquette, France). Next, 4 µL of 1:50 diluted cDNA template were amplified using the qPCR assay kit, following the manufacturer’s instructions (Biorad, Marnes-la-coquette, France) using SYBR Green dye to measure duplex DNA formation. The sequences of primers used in the present study are given in Table 1.

2.6 Statistical analysis
Paired t-test was performed to determine whether there were differences between the groups. \( P < 0.05 \) was considered significant.

3 Results

3.1 Loss of LXR results in perturbations of lipid content of the caput epididymidis
Histological analysis using oil red O staining performed on frozen sections showed an abnormal accumulation of neutral lipids in vacuoles observed in the epithelium and peritubular tissues of \( \text{lkr} \beta^- \) and \( \text{lkr} \alpha; \beta^- \) mice (Figure 1C, D), whereas no increased oil red O staining was observed in the wild-type and \( \text{lkr} \alpha^- \) deficient mice (Figure 1A and B, respectively). Because a cholesteryl ester accumulation has already been described in the LXR\( \beta^- \)-deficient mice [9], we hypothesized that the peripheral staining in the epididymis was specific for smooth muscle. In the \( \text{lkr} \alpha^- \) mice, we observed a low accumulation of oil red O, specifically in the epithelial compartment, with the appearance of infiltrated cells, which could be macrophages (Figure 1B, higher magnification, arrows): a hypothesis that is currently being investigated.

To determine the nature of lipids accumulated in the epididymis, HPTLC analyses were performed on whole lipid extracts from the caput epididymidis in the four genotypes. Although LXR-mediated tri-acylglycerol ac-
accumulation has already been reported in vascular smooth muscle cells [10], chromatography assays revealed that only the fraction containing cholesteryl esters was increased (Figure 2A). Densitometric analysis of the chromatograms confirmed this observation: mean cholesteryl esters contents were significantly increased by 12 and 25-fold in \( \text{lxr}^\beta^{-/-} \) and \( \text{lxr}^\alpha\beta^{-/-} \) mice, respectively (Figure 2B, \( P < 0.05 \) compared with wild type), whereas \( \text{lxr}^\alpha^- \) mice presented the same low amount of cholesteryl esters as the wild-type mice. Surprisingly, it seems that the lack of both types of LXR induces a more severe accumulation of cholesteryl esters, but the observed difference was not statistically significant (\( P = 0.2 \)). No significant difference in free cholesterol and phospholipid contents was observed among the four different genotypes (Figure 2B).

The results led us to conclude that the phenotype was a result of the absence of LXR\( \beta \). LXR\( \alpha \) does not have a redundant function in this part of the epididymis, despite its expression (Figure 3).

3.2 Loss of LXR does not modify fatty acid contents in

Table 1. Sequences of the primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>((\text{accession #}))</th>
<th>(5') sequences</th>
<th>Size of the amplicon (bp)</th>
<th>Reference</th>
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<tr>
<td>(\text{lxr}^\alpha)</td>
<td>(AJ_132601)</td>
<td>GGG AGG AGT GTG TGC TGT CAG</td>
<td>192</td>
<td>Mouzat et al. [9]</td>
</tr>
<tr>
<td>(\text{lxr}^\beta)</td>
<td>(NM_009473)</td>
<td>AAG CAG GTG CCA GGG TGC T</td>
<td>140</td>
<td>Mouzat et al. [9]</td>
</tr>
<tr>
<td>(\text{srebp}^1c)</td>
<td>(NM_011480)</td>
<td>TGC ATT CTG TCT GTG TGT</td>
<td>189</td>
<td>Mouzat et al. [9]</td>
</tr>
<tr>
<td>(\text{scd}^1)</td>
<td>(NM_009127.2)</td>
<td>TAG CCT GTA AAA GAT TTC TGC AAA CC</td>
<td>89</td>
<td>Present study</td>
</tr>
<tr>
<td>(\text{scd}^2)</td>
<td>(NM_009128.1)</td>
<td>TGC CTT GTA TGT TCT GTG G</td>
<td>219</td>
<td>Present study</td>
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<td>(BC 023632)</td>
<td>GTT GAG GGC AA T GCC AGC CCC</td>
<td>358</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Figure 2. Liver X receptor \( \beta \) (LXR\( \beta \))-deficient mice have a higher level of cholesteryl esters. (A): Chromatogram of high-performance thin-layer chromatography (HPTLC) analysis of lipids extracted from the caput epididymidis from the four genotypes. The results are representative of three independent experiments in triplicate for each genotype. (B): Densitometric analysis of HPTLC assays. The chromatograms were scanned, and spots were quantified, as described in the Material and methods section. Standard dosage values gave curves with linear-regression coefficients \( (R^2) \) of 0.90 or greater. \( ^* P < 0.05 \), compared with wild-type (WT) mice. PE, phosphatidylethanolamine; PC, Phosphatidylcholine; SM, Sphingomyelin.
Liver X receptors and epididymis

Figure 3. Genes involved in fatty acid metabolism are basally lower expressed in liver X receptor β (LXRβ)-deficient mice. Histograms representing quantitative polymerase chain reaction performed on samples obtained from the caput epididymidis from the four genotypes. Results are presented as fold-activation (of the ratio over gapdh) compared with wild-type (WT) mice for each gene investigated, and are expressed as means ± SEM from triplicate assays from three different individual caput epididymidis for each genotype. b P < 0.05, compared with wild type.

the caput epididymidis

Because LXR are known to regulate the expression of srebp1c and fas, encoding the sterol response element binding protein and fatty acid synthase, respectively, the fatty acid profile of phospholipids and neutral lipids was determined after lipid extraction and separation in the caput epididymidis from the four genotypes. Samples were separated by gas chromatography and the peaks obtained were compared and integrated against known fatty acids mixes. The overall results revealed no significant difference in the fatty acid composition among the genotypes in all the studied samples (Figures 4 and 5).

3.3 Loss of LXRβ in the caput epididymidis modifies the basal levels of genes involved in fatty acid synthesis

The results obtained for the fatty acid analysis raised the question of whether the deficiency of LXR could influence fatty acid metabolism in the caput epididymidis. Stearoyl Co-A desaturases 1 and 2 (scd1 and scd2) are indirect LXR target genes in other tissues, via the activation of SREBP-1c [11, 12]. They are also known to be expressed at high levels in the caput epididymidis in mice and rats [13]. These genes encode enzymes responsible for the desaturation of fatty acids, mainly palmitic (C16:0) and stearic acid (C18:0), to produce palmitoleic (C16:1 n-7) and oleic (C18:1 n-9) acids involved in the production of triglycerides, or incorporated in the cholesteryl esters. This point is interesting, as we previously demonstrated an increase in the cholesteryl esters contents in lxrβ−/− and lxrαβ−/− mice. Expression of srebp-1c was also investigated.

Figure 3 clearly shows that the levels of scd1, scd2 and srebp1c were significantly lower in lxrβ−/− and lxrαβ−/− mice (P < 0.05). This result is in accordance with the fact that the three genes are LXR-regulated. However, the downregulation of scd1 and scd2 expressions does not correlate with the increase in the cholesteryl ester content observed in the same genotypes.

Therefore, it appears that LXRβ is the predominant isoform regulating the lipid metabolism in caput epididymidis. The presence of LXRα alone does not compensate for the lack of LXRβ, as demonstrated before for the changes in the cholesteryl esters levels.

4 Discussion

Lipid homeostasis in the epididymis is an important biological process as the modifications occurring during sperm transit in this organ are fundamental for the fertilizing capacities of the male gamete. This lipid maturational process is accompanied by other maturational events at the protein and biochemical levels. However, the pre-
Figure 4. Fatty acid profile analysis of phospholipids from the caput epididymidis from the four genotypes. Histogram representing the different classes of fatty acids in the phospholipids extracted from the caput epididymidis from the four different genotypes and separated by gas chromatography. Results are presented as the percentage of total fatty acids for each individual category, and are expressed as means ± SEM from three different determinations. WT, wild type.

Figure 5. Fatty acid profile analysis of neutral lipids from the caput epididymidis from the four genotypes. Histogram representing the different classes of fatty acids in the neutral lipids extracted from the caput epididymidis from the four different genotypes, separated by gas chromatography. Results are presented as the percentage of total fatty acids for each individual category, and are expressed as means ± SEM from three different determinations.
cise molecular mechanisms underlying the epididymal lipid maturation process are so far not very well known. Interestingly, male mice deficient in the two LXR isoforms presented an epididymal phenotype and became progressively infertile between the ages of 5 and 9 months. This phenotype was not observed in the animals deficient in only one isoform of these nuclear receptors. As LXR are well known regulators of cholesterol and fatty acid metabolism by their target genes, the aim of the present work was to investigate the cholesterol and fatty acid composition of the caput epididymidis from the wild-type, \( \text{lxr}^\alpha/-, \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) mice, as well as the levels of some genes involved in the fatty acid metabolism, such as \( \text{scd1}, \text{scd2} \) and \( \text{srebp1c} \).

The lack of LXRβ leads to an abnormal accumulation of cholesteryl esters in the epithelium and the peritubular tissues in the epididymis. The peritubular staining might be related to lipid accumulation in the smooth muscle cells surrounding the epididymal duct. Indeed, it was recently demonstrated that uterine smooth muscle cells presented such accumulations in \( \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) female mice. This was associated with a significant decrease in the function of these cells, as shown by a reduction in the contractile activity of the uterus: they were less responsive to higher concentrations of oxytocin and a PGF2α analogue, two efficient stimulators of uterine contraction [9]. It seems that cholesterol loading of smooth muscle cells provokes a reduction in the contractile ability of these cells. In the caput epididymidis, it has been shown that, from a histological point of view, some tubule sections lack any luminal content, whereas other tubule sections are filled with amorphous substances, with very few visible spermatozoa, in the \( \text{lxr}^\alpha\beta/- \) mice [5]. This is quite similar to the granuloma formation occurring after vasectomy or under certain pathological conditions, such as cholesterol granuloma in human or high dose-testosterone implants in mice [14–15]. This accumulation of unknown amorphous material is not visible in the data presented here because the individuals used in the present study were younger than those presenting the phenotype described by Frenoux et al. [5] (7 vs. 11 months). However, one can hypothesize that, as seen in the uterus, the smooth muscle cell accumulation of lipids impairs the contractile function of these cells, thus favoring the appearance of this amorphous substance. Furthermore, the lipids accumulated were determined to be cholesteryl esters, which are significantly raised in the caput epididymidis from \( \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) mice. Once again, this situation is similar to what was observed in the uterus: only the fraction containing cholesteryl esters was significantly increased, after normalizing to uterus weight, at 3 and 12 months of age in \( \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) female mice, compared with wild-type mice. In both the caput epididymidis and the uterus, the increase in oil red O staining observed in the \( \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) mice was a result of the accumulation of cholesteryl esters. This point supports the fact that LXR-dependent regulation of lipid metabolism is a crucial biological process in the male as well as in the female reproductive tract.

During epididymal transit, the phospholipids and the fatty acid composition of the sperm cell membrane is modified. These changes include a loss of 25%–48% of the total phospholipids, with different changes among the individual classes. An increase in the relative percentage of polyunsaturated fatty acids occurs with, for example, palmitic acid (C16:0) being the major phospholipid-bound fatty acid of immature spermatozoa, whereas docosahexaenoic acid (C22:6) is predominant in mature cells [16]. This indicates an active fatty acid metabolism in the epididymis, and considering the influence of LXR on fatty acid metabolism and the modification of the lipid composition observed in the knockout mice, it seemed interesting to analyze the fatty acid composition of the caput epididymides. Surprisingly, no significant difference was detected in the fatty acid composition of the phospholipids extracted from total epididymal tissues. We also did not notice any difference in the neutral lipid fraction, whereas the cholesteryl esters were higher in \( \text{lxr}^\beta/- \) and \( \text{lxr}^\alpha\beta/- \) mice. We can explain this result using several hypotheses: first, lipids were extracted from whole epididymal tissue, which means that modifications of the sperm composition, as a result of epithelial dysfunction, might not appear in this overall lipid extract. Therefore, it is crucial that these analyses on lipids extracted from isolated spermatozoa in the four different genotypes be repeated. The main obstacle in mice (compared to humans, for example, where ejaculated spermatozoa can be obtained) is having enough biological material to perform the assays, as we must recover spermatozoa from the cauda epididymidis. Therefore, the sperm recovery technique must be improved to obtain sufficient sperm cells from a limited number of animals to perform these analyses. Second, we could expect modification of the fatty acids in the neutral lipids, as the increase in cholesteryl esters should be associated with an increase in fatty acids esterifying the cholesterol molecules (i.e. mainly palmitoleic acid [C16:1n-7] and oleic acid [C18:1n-9]) [17]. As indicated, these fatty acids are not modified in the deficient compared with wild-type mice, and we can observe very high inter-individual variations. This is probably due to the low amount of neutral lipids obtained from a single caput epididymidis, as the majority of fatty acids appear in very low quantity. This point will need to be answered by performing the
same assays on a pool of tissues from the wild-type and the different invalidated mice.

Although we cannot draw any final conclusion on the fatty acid modifications in the caput epididymides of the lxrβ-/- mice, it is well known that fatty acid metabolism is an LXR-regulated biological process [18]. Therefore, gene expression analysis was undertaken by qPCR on known LXR-regulated genes: srebp-1c, scd1 and scd2. SREBP are a family of transcription factors that are involved in the regulation of cholesterol homeostasis and fatty acid biosynthesis and uptake. SREBP-1c preferentially controls the transcription of fatty acid biosynthetic genes (see [19] for a review). scd1 is a target gene of SREBP-1c in different tissues [11, 12] and mice deficient for scd1 were shown to have a reduced ability to esterify cholesterol for hepatic storage [20]. Furthermore, scd1 and scd2 are expressed at high levels in the rat epididymis, even higher than in the liver [13]. Considering the cholesteryl ester accumulation observed in the caput epididymides of the lxrβ-/- and lxrαβ-/- mice, one could expect a modification of the expression level of these genes. Surprisingly, all three genes were downregulated in the caput epididymides of the lxrβ-/- and lxrαβ-/- mice (scd2 being only downregulated in the lxrαβ-/- genotype). These data indicate that the observed accumulation of cholesteryl esters cannot be correlated with a greater synthesis of esterified fatty acids, which is in accordance with the fatty acid analysis. However, these results are in accordance with the fact that srebp-1c and its own target genes are LXR-regulated, and they support the fact that the regulation mechanisms in the caput epididymides are under the control of the LXRβ isofrom. The explanation concerning the cholesteryl esters accumulation in the caput epididymides of the lxrβ-/- and lxrαβ-/- mice needs to be more deeply investigated to bring new elements into lipid homeostasis in this organ. It will also be of primary interest to determine the fatty acid and sterol composition of the sperm cells in the context of the different LXR genotypes to establish relationships between LXR-dependent regulations and putative fertility problems, such as altered sperm cell lipid composition in humans, which can be associated with infertility [21].

The present paper demonstrates that LXR are important regulators of cholesterol and fatty acid metabolism in the epididymis, and that this function is firmly associated with the LXRβ isofrom.

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References

Liver X receptors and epididymis


The ~Omes are coming! The ~Omes are coming! Research on the epididymis in the 21st century

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Looking at the history of epididymal research provides a level of confidence in predictions of cause and effect that looking at the future cannot achieve. Thus, when one of us (Hamilton, 2002) wrote a short essay on the history of the testicular excurrent ducts it became fairly obvious that advances in understanding the epididymis followed advances in other fields, particularly those that provided new instrumentation to probe at ever finer levels of detail. Can this same theme lead to predictions about what will happen in epididymis research over the next century? We think so. Thus, this essay will explore the powerful new trends in research encompassed by the suffix ~ome (genome, proteome, etc.) that are permeating biological science, and will discuss the technical needs that will arise as analysis becomes more refined. It also will make some predictions about the types of scientists who will be necessary to compete successfully in this new era, and will end by asking the provocative question of: Who will actually prove that what the ~omics are discovering is true? In other words, who will do the “grunt” work?

Note: This is abstract of the final lecture in Epid IV, which was given by David Hamilton (Mineapolis, MA, USA) on December 7th. As he could not attend the meeting, he provided it as a video presentation. His talk entitled “The ~Omes are coming!” illustrated what, from his point of view, would be epididymal research in the 21st century.

Video clips of this lecture can be found on the internet at http://www.asiaandro.com/1008-682X/9/v9i4.htm