Chapter V

Effect of HDL on LPS-mediated TNF-α production in adipocytes
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Introduction

The word “inflammation comes from the Latin word “inflammo” meaning “I ignite” or “I set alight”. Many words related to “burning” are derived from this Latin word, like ‘inflammable’ meaning combustible. The symptoms of inflamed tissues give the impression of burning since they include pain, redness, heat and swelling.

Inflammation is not a harmful process. It is part of the body’s innate immune response to injury or pathogen entry. Initially inflammation is beneficial since its aim is to remove the causative agent. However it can lead to harm by producing more inflammatory responses to existing inflammation. The inflammation hypothesis of cardiovascular diseases suggests that the cardiovascular diseases are because of inflammation. The build up of fatty streak in the blood vessel can trigger an immune response which leads to inflammation.

The innate immune system which is the first line of defence of the body has many different mechanisms to get rid of the potentially dangerous cells or molecules. It was believed that the function of the immune system is to distinguish ‘self’ from ‘nonself’ and then get rid of ‘non self’. However this concept fails to explain many anomalies of the immune system. The current concept, describes the function of the immune system, as one which can identify patterns. The pathogen associated pattern recognition system is quite elaborate and includes molecules and cells that would have undergone modification due to oxidation, glycation or some other modification.

The pathogen associated pattern recognition system makes use of specific pattern identification receptors on the surface of phagocytic cells. These receptors are called Toll-like receptors (TLRs). They receive their name from their similarly to the protein coded by the Toll gene of Drosophila (Hansson GK and Edfeldt K 2005). The Toll gene when mutated made the drosophila look highly unusual. It is said that the discoverer of this gene, Nusslein-Volhard and her colleagues explained in German “Das ist ja toll” meaning "that’s great!" and hence this gene got its name “Toll”. TLRs are single membrane-spanning, non-catalytic receptors usually expressed on cells such as macrophages and dendritic cells. They are a family of receptors involved in pattern recognition and can recognize molecules derived from microbes generally
called as endotoxins. These endotoxins are now used synonymously with the term ‘lipopolysaccharide’ (LPS) which are the major constituent of outer cell membrane of Gram –ve bacteria (Rietschel ET et al 1994). Lipopolysaccharides are the major components of the outer membrane of Gram –ve bacteria and are the endotoxin of these microorganisms. The structure of the bacterial lipopolysaccharide is shown in figure 5.1.

**Figure 5.1 Structure of Lipopolysaccharide from Gram Negative Bacteria** ([microbeonline.com](http://microbeonline.com))

The signalling pathway generated by TLRs to bacterial antigens is shown in Figure 5.2
Figure 5.2 Signalling by Toll-Like Receptors in phagocytic cells. (From KEGG pathways. www.genome.jp/kegg-bin)
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The lipoproteins of Gram Positive bacteria bind and activate TLR 1 and 2 whereas lipopolysaccharides (LPS) of Gram Negative bacteria activate TLR 4. The downstream effects and the final end result of activating these receptors are also different. However some of the end products are common to these agonists. For example both lipoproteins and LPS ultimately produce proinflammatory cytokines.

LPS is recognised by Toll like receptors 4 (TLR 4) which interacts with other different extracellular proteins; LPS binding protein (LBP), CD 14 and myeloid differentiation protein-2 (MD-2) to induce a signalling cascade leading to the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and production of proinflammatory cytokines. This scheme is summarized in Figure 5.3

![Figure 5.3 Signal transduction induced by bacterial LPS.](image)

In this chapter we have investigated the ability of HDL and oxidized HDL to bind to bacterial LPS and modulate the effect of LPS on TNF-α (tumor necrosis factor alpha) production in adipocytes.
Materials and Methods

Lipopolysaccharide (LPS) was purchased from sigma Aldrich USA, Rat TNF-α ELISA kit was from Gen-Probe Inc. USA. All other chemicals were of analytical grade purchased from SRL Chemicals and HiMedia, India.

Preparation of HDL and LDL.

Human serum HDL was isolated by density gradient ultracentrifugation as described in Chapter II.

Oxidation of HDL by AAPH

2 mg of HDL protein in phosphate buffer containing 1mM EDTA or 4mM AAPH or both was incubated for 3 hrs at 37°C followed by incubation with addition of 5µl of 2mg/ml DCFH and RFI was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A sensitivity level of 0.1 and slit widths of 2.5 and 10 nm were used for excitation and emission, respectively (Nakano T and Nagata A 2003, Garner B 1998).

Isolation of adipose tissue

Male Wister rats weighing 200-250 were utilized. These were housed in single cages were given rat chow and water ad libitum. Rats were killed by exsanguination under diethyl ether anaesthesia. The epididymal fat pads were removed under sterile conditions and placed in 0.9% saline in a petridish for removal of the epididymal fat pad from the border of the epididymis and used immediately.

Stimulation of Adipose tissue

The fat pads were cut in to small pieces, weighed and incubated in HBSS (Hank’s Balanced Salt Solution) containing LPS/HDL/ox-HDL for different time intervals at room temperature and each aliquots were collected at each intervals of time, kept in -20°C and later used for estimation of TNF-α.

TNF-estimation

Estimation of TNF-α was carried out using Rat TNF-α ELISA KIT from Gen-Probe Inc. and protocol was followed provided by the kit.
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Results

The time course of TNF-α production increased with time and reached a maximum at 16-24 hrs. Both LPS and HDL showed the same amount of TN-α production (Figure 5.4)

![Figure 5.4. Time course of TNF α production by LPS and HDL in Adipose tissue](image)

LPS (1ug) or HDL (2 mg protein) were incubated with adipose tissue in vitro as described in the methods. Aliqotes were taken for TNF-α determination.

Time course of TNF-α production by LPS in the presence of HDL and ox-HDL is shown in Figure 5.5.

![Figure 5.5. Time dependent production of TNF-α in the presence of HDL and Ox-HDL](image)

LPS (1ug) was incubated with HDL or Ox-HDL (2 mg protein) for upto 24 hrs. Aliqotes were taken for TNF-α determination as described in the methods.
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There was a time dependent TNF-α production. However similar time dependent TNF-α production was not seen in the case of oxidized HDL. In fact at 24 hrs there was a decrease in the production of TNF-α by ox-HDL.

TNF-α produced by Adipocytes in response to various agonists like LPS, HDL and ox-HDL is shown in figure 5.6

![Figure 5.6](image_url)

Figure 5.6. TNF-α production in adipose tissue in vitro in the presence of various agonists.

Adipose tissue was incubated in vitro in the presence of different agonists as described in the methods. Aliquots were taken at the end of 24 hrs to determine TNF-α in the medium.

HDL and Ox-HDL produced the same amount of TNF-α as LPS. However, LPS produced maximum TNF-α when present in combination with HDL but not ox-HDL.
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Discussion

Adipose tissue is not only a storage tissue for lipids but is also an endocrine organ (Flier 1995). It is capable of producing TNF-α which regulates almost every aspect of adipose biology (Sethi and Hotamisligil 1999). It is also emerging to be a key component of metabolic diseases such as obesity, diabetes and CVD.

Adipocytes are recognized to possess significant inflammatory properties like macrophages they are sensitive to infectious agents and cytokine mediated inflammatory signals. Adipocytes express TLR-4 when stimulated by LPS; these receptors activate NF-κB signal transduction pathways (Berg et al 2004).

In our studies we found only 1.8 fold stimulation in TNF-α production over the control in the presence of 1µg of LPS. In the in vivo situation LPS stimulates TLR-4 through interaction with two other proteins LPB (lipopolysaccharide binding protein) and CD 14. However in the in vitro experiment we did not provide these proteins and hence there may not have been a large response. However in the presence of native HDL but not oxidized HDL there was 2.4 fold stimulation in the TNF-α production over LPS alone. The pathway of TNF-α production by LPS is shown in figure 5.7.

Figure 5.7. Signaling pathways involved in LPS induced TNF-α production in human adipocytes.
LPS, with LBP and CD14 bind to TLR4 on mature human adipose cells. Binding activates two main pathways in the cells. One pathway leads to the activation of NF-kappaB (NF-kB) (through TRAF6 and IKK). The other pathway passes through phosphorylated p38 MAP Kinase. Both pathways enable the activation of TNF-α transcription, followed by cleavage of the protein via a membrane metalloprotease, ADAM17 or TACE, leading to the release of the soluble form of TNF-α. The PI3K represents a third pathway, which activates NF-kB and p38 MAPK. Another kinase, maybe the PI4K, plays an inhibitory role in the LPS activation of these two pathways. As far as the PKC is concerned, it probably activates IKK, or the p38 MAPKs, or both pathways. However, activation is only visible once the PI4K is inactive. It is therefore possible that PI4K constitutively inhibits PKC. Interestingly oxidized HDL was shown to down regulate CD36 a member of the class B scavenger receptor family member by a p38 MAPK mechanism (Ren et al 2010). If p38 is down regulated, then it would also down regulate the LPS mediated signalling response. However why native HDL stimulated the LPS mediated TNF-α production is not known.

The antiinflammatory effects of HDL imply that it should have bound the bacterial LPS and neutralized its effect; on the contrary the effects were enhanced. It is possible that the mildly oxidized HDL may provide a co-stimulatory signal for the action of LPS thus synergistically enhancing the production of TNF-α. However highly oxidized HDL may not be able to provide this co-stimulatory signal and may actually down regulate the stimulatory signal of LPS.

Studies that have shown binding of LPS to HDL, have used whole blood, and incubated the LPS with whole blood. The LPS was bound to HDL and this was taken to suggest that HDL binds to LPS and makes it unavailable for the macrophages to act (Feingold and Grenfield 2011). There are older reports which show that pyrogenicity of LPS-HDL complex is lower (Baumberger et al 1991). Also reconstituted HDL reduced LPS-stimulated TNF-α production (Casas et al 1995). These studies were carried out in cultured cells and not in intact Adipocytes.
Conclusion

Our results suggest that the HDL potentiated the action of LPS on adipocyte tissue and stimulated the production of TNF-α by 2.4 folds whether this is a beneficial reaction or harmful reaction is not known at present.
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References


