Reciprocal and Coordinate Regulation of Serum Amyloid A Versus Apolipoprotein A-I and Paraoxonase-1 by Inflammation in Murine Hepatocytes

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Objectives—During inflammation, the serum amyloid A (SAA) content of HDL increases, whereas apolipoprotein A-I (apoA-I) and paraoxonase-1 (PON-1) decrease. It remains unclear whether SAA physically displaces apoA-I or if these changes derive from coordinated but inverse transcriptional regulation of the HDL apolipoprotein genes. Because cytokines stimulate the hepatic expression of inflammatory markers, we investigated their role in regulating SAA, apoA-I, and PON-1 expression.

Methods and Results—A cytokine mixture (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, and IL-6) simultaneously induced SAA and repressed apoA-I and PON-1 expression levels. These effects were partially inhibited in cells pretreated with either nuclear factor κB (NF-κB) inhibitors (pyrrolidine dithiocarbamate, SN50, and overexpression of super-repressor inhibitor κB) or after exposure to the peroxisome proliferator-activated receptor-α (PPARα) ligands (WY-14643 and fenofibrate). Consistent with these findings, the basal level of SAA was increased, whereas apoA-I and PON-1 decreased in primary hepatocytes from PPARα-deficient mice as compared with wild-type mice. Moreover, neither WY-14643 nor fenofibrate had any effect on SAA, apoA-I, or PON-1 expression in the absence of PPARα.

Conclusion—These results suggest that cytokines increase the expression of SAA through NF-κB transactivation, while simultaneously decreasing the expression of apoA-I and PON-1 by inhibiting PPARα activation. Inflammation may convert HDL de novo into a more proatherogenic form by coordinate but inverse transcriptional regulation in the liver, rather than by physical displacement of apoA-I by SAA. (Arterioscler Thromb Vasc Biol. 2006;26:1806-1813.)

Key Words: high-density lipoprotein ■ serum amyloid A ■ NF-κB ■ PPARα ■ apolipoprotein A-I

Chronic low grade elevation of inflammatory markers such as C-reactive proteins (CRP) and serum amyloid A (SAA) are associated with increased cardiovascular risk.1,2 SAA are apolipoproteins that are transported predominantly in high density lipoprotein (HDL).3,4 SAA also have a carboxy-terminal proteoglycan binding domain, which may facilitate lipoprotein retention by proteoglycans in the vascular intima, thus potentially being atherogenic.5 SAA is synthesized primarily in the liver, where expression increases in response to cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α.7

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HDL plays a critical role in reverse cholesterol transport, which is believed to account for much of the atheroprotective effect of HDL.8,9 Cholesterol removal from cells via the ABCA1 pathway is mediated primarily by apolipoprotein A-I (apoA-I),10,11 HDL levels decrease during acute inflammation12-15 and are low in chronic conditions associated with increased levels of inflammatory markers.16 ApoA-I expression is inhibited by inflammatory stimuli.17,18 Paraoxonase-1 (PON-1) is another HDL apolipoprotein.19 PON-1 is considered to be atheroprotective and decreases after inflammatory stimuli.14,20,21 HDL from mice exposed to cytokines has impaired capacity to inhibit low-density lipoprotein (LDL) oxidation and protect against monocyte adherence.22 Little is known about the hepatic regulation of PON-1 expression in relation to apoA-I and SAA. Isolated SAA has been shown to displace apoA-I and PON-1 from HDL in vitro.4,23,24 However, because apoA-I expression can be inhibited by inflammatory stimuli,17,18 it is possible that alterations in HDL apolipoprotein composition during inflammation are the result of changes in the hepatic regulation of these apolipoproteins, rather than caused by physical displacement of apoA-I or PON-1 by SAA. Moreover, it is unknown whether the expression of apoA-I, PON-1, and SAA are regulated independently or coordinately.

The current study was undertaken to evaluate whether proinflammatory cytokines coordinately regulate the expres-
sion of SAA, apoA-I, and PON-1 in hepatocytes. We demonstrate that cytokines coordinately increase SAA and decrease apoA-I and PON-1. These reciprocal changes are promoted by nuclear factor κB (NF-κB) and repressed by the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) in a manner dependent on both these key transcriptional mediators. These observations are intriguing given that neither apoA-I nor PON-1 promoters contain NF-κB sites, and the SAA promoters do not contain PPAR response elements (PPRE), further supporting the coordinated regulation of these HDL apolipoproteins.

Materials and Methods

Cell Culture and Transfection

The murine hepatoma cell line Hepa 1–6 was cultured in DMEM supplemented with 10% FBS. The murine AML12 and NMH hepatocyte cell lines were cultured in DMEM-Ham F-12 medium supplemented with 10% FBS, ITS solution (BD Biosciences), and 0.1 mM L-dexamethasone. Hepa 1–6 cells were transiently transfected with a CMV-ΔN-κB expression vector that overexpresses a nonphosphorylatable N-terminal deletion mutant of inhibitor κB (IκB) and LacZ plasmids using Fugene 6 (Roche). After 24 hours, cells were exposed to a mixture of cytokines (IL-6, IL-1β, and TNF-α, all at 10 ng/mL; R&D Systems) ±other reagent(s). Cells were harvested after the indicated times, and the results were normalized to LacZ expression to control for transfection efficiency.

Mouse hepatocytes were isolated from 8- to 10-week-old 129S1/SvImJ PPARα-deficient mice as described.

Animals

To study the effect of inflammation on the hepatic regulation of apoA-I, PON-1 and SAA in vivo, C57BL/6 mice received an intraperitoneal (ip.) injection of lipopolysaccharide (LPS) (0 to 100 μg/mL; R&D Systems) ±other reagent(s). Cells were harvested after the indicated times, and the results were normalized to LacZ expression to control for transfection efficiency.

To study the role of PPARα in the regulation of apoA-I, PON-1, and SAA, wild-type C57BL/6 (WT) and PPARα-deficient mice as described.

Results

Cytokine Stimulation of Hepatocytes Increases SAA Expression While Simultaneously Decreasing ApoA-I and PON-1 Expression

Cytokines such as IL-6, IL-1β, and TNF-α increase the hepatic expression of SAA. Because inflammation reduces HDL concentrations, we examined the effect of cytokines on the simultaneous secretion of SAA and apoA-I by cultured hepatocytes. Because in vivo conditions involve exposure to multiple cytokines, the murine hepatoma cell line Hepa 1–6 was stimulated with a mixture of cytokines known to be key signaling molecules in atherogenesis, namely IL-6, IL-1β, and TNF-α. Cytokine stimulation significantly increased SAA protein, detected by immunoblotting, in the media of Hepa 1–6 cells, whereas the amount of apoA-I simultaneously decreased (Figure 1A). A suitable antibody was not available for PON-1. Consistent with the changes in protein levels, cytokine stimulation increased SAA mRNA expression in Hepa 1–6 cells in a time-dependent fashion. Conversely, apoA-I and PON-1 expression were both decreased in Northern blot analysis of Hepa 1–6 cells (Figure 1B). Similar effects were observed in both AML12 and NMH hepatocytes, with cytokine stimulation increasing SAA expression and decreasing expression of apoA-I and PON-1 (supplemental Figure I, available online at http://atvb.ahajournals.org).
cytokine regulated SAA, apoA-I, and PON-1 expression independently. However, a combination of all three cytokines showed more potent effects and was used for all further experiments \(^7\) (supplemental Figure II).

To determine whether inflammation resulted in similar changes in vivo, LPS (0 to 100 \(\mu\)g/mouse) was injected ip in C57BL/6 mice for hepatic mRNA analysis by Northern blot 24 hours later. Consistent with the in vitro data, SAA expression increased, whereas apoA-I and PON-1 expression decreased dose-dependently (Figure 1D). To exclude a direct effect of LPS on the regulation of these genes in hepatocytes, LPS was added directly to AML12 cells for 24 hours. Neither SAA or apoA-I expression levels were changed by LPS treatment, while the mixture of cytokines regulated these genes (supplemental Figure III). These observations imply that cytokines produced in response to LPS stimulation of nonparenchymal liver cells, in particular, Kupffer cells or extrahepatic macrophages, were stimulated by LPS in vivo, which in turn activated the hepatocytes. These results are consistent with previous observations. \(^{36,37}\)

To investigate whether the mitogen-activated protein (MAP) kinase signal transduction pathway was involved in these cytokine-mediated effects on the SAA, apoA-I, and PON-1 genes, the MEK inhibitors, PD98059 (50 \(\mu\)mol/L) and U0126 (10 \(\mu\)mol/L), which inhibit extracellular signal regulated kinase (ERK) pathway, were added to LPS in vivo, which in turn activated the hepatocytes. These results are consistent with previous observations. \(^{36,37}\)

NF-\(\kappa\)B Inhibitors Block the Cytokine Effects on SAA, ApoA-I, and PON-1 mRNA Levels

NF-\(\kappa\)B binding sites are present on the promoters of the acute phase form of SAA produced by liver, SAA1 and 2, \(^{7}\) but not on the promoters of either apoA-I or PON-1. \(^{25,27}\) To evaluate the involvement of the NF-\(\kappa\)B pathway in the cytokine-induced changes in SAA, apoA-I, and PON-1 expression, the NF-\(\kappa\)B inhibitors PDTC (a universal NF-\(\kappa\)B inhibitor) and SN50 (a peptide inhibitor of NF-\(\kappa\)B translocation) were added to Hepa 1–6 cells concurrently with the cytokine mix. PDTC partially inhibited cytokine-induced SAA secretion and partially restored apoA-I secretion (Figure 2A). Moreover, SN50 partially inhibited the increase of SAA mRNA expression and partially reversed the suppression of apoA-I and PON-1 mRNA expression evoked by the cytokines (Figure 2B).

The I\(\kappa\)B Super-Repressor Inhibits the Reciprocal Effect of Cytokines on SAA, and ApoA-I and PON-1

I\(\kappa\)B is a well-established endogenous repressor of NF-\(\kappa\)B. To further study the role of NF-\(\kappa\)B in the effects of cytokines on SAA, apoA-I, and PON-1, a super-repressor expression vector for I\(\kappa\)B was transiently transfected into Hepa 1–6 cells. CMV-\(\Delta N-I\kappa B\) express an I\(\kappa\)B super-repressor under the control of the cytomegalovirus (CMV) promoter, where the N-terminal deletion mutant of I\(\kappa\)B prevents the phosphorylation that is necessary for it to dissociate from NF-\(\kappa\)B. \(^{30}\) Overexpression of the I\(\kappa\)B super-repressor by transient trans-
PPARα Agonists Reverse the Effect of Cytokines on SAA, ApoA-I, and PON-1 Expression

The ligand-activated transcription factor PPARα limits inflammation via effects on NF-κB activation.38,39 Moreover, PPARα can induce apoA-I expression. Therefore, we examined the effects of the PPARα ligands WY-14643 and fenofibrate (CalBiochem) on cytokine-mediated expression of apoA-I and PON-1, both of which are known to have PPRE in their promoters,25,27 and SAA, which does not. WY-14643 blocked the cytokine-mediated secretion of SAA and restored apoA-I secretion (Figure 5A). Both WY-14643 and fenofibrate reversed these cytokine effects, inhibiting the increase in SAA mRNA expression and ameliorating the suppression of apoA-I and PON-1 mRNA expression (Figure 5B). Thus, PPARα activation antagonizes cytokine-induced NF-κB activation in hepatocytes, increasing apoA-I and PON-1 and decreasing SAA expression. However, in the absence of cytokines, fenofibrate did not increase apoA-I mRNA expression in either cell lines, whereas WY-14643 led to a slight increase in apoA-I mRNA.

Because both WY-14643 and fenofibrate could potentially act independently of PPARα, the effects of these PPARα agonists was tested in hepatocytes isolated from PPARα-deficient mice. Primary cultures of hepatocytes from PPARα−/− and PPARα+/− mice were exposed to cytokins either with or without WY-14643 (100 μmol/L) or fenofibrate (100 μmol/L). Interestingly, hepatocytes from PPARα−/− mice expressed high levels of SAA and low level of apoA-I and PON-1 at baseline as compared with wild-type animals (Figure 6). Neither WY-14643 nor fenofibrate had any effect on SAA, apoA-I, or PON-1 in PPARα−/− mice, either in the presence or absence of cytokines (Figure 6). In primary hepatocytes from the wild-type control mice, the effect on SAA, apoA-I, and PON-1 was the same as seen in the other cell types (Figure 6A). Also, neither WY-14643...
nor fenofibrate alone upregulated apoA-I expression. To investigate the NF-κB transactivation in both PPARα+/+ and PPARα−/− mouse livers, EMSA was performed (Figure 6B). Compared with PPARα+/+ mice, the level of NF-κB transactivation was high in PPARα−/− mice in the basal state (compare lane 5 with lane 1). Treatment with WY-14643 blocked NF-κB transactivation in PPARα+/+ mice, whereas treatment with WY-14643 in PPARα−/− mice did not inhibit NF-κB transactivation (compare lanes 3 and 4 with lanes 7 and 8). These gel shift bands appear specific because formation of the complex was blocked with either an unlabeled oligonucleotide, but not a mutant NF-κB oligonucleotide. These results suggest that PPARα agonists limit cytokine-induced changes in SAA, apoA-I, and PON-1 in a PPARα-dependent manner, perhaps by modulating NF-κB activation.

The determination of whether a mixture of cytokines can regulate either NF-κB and PPARα expression, the protein levels of these nuclear transcription factors in lysates of cells treated with or without cytokines were examined by Western blot. After 24-hour exposure to cytokines, the expression levels of both nuclear factor did not demonstrate much change (supplemental Figure IV).

**Discussion**

We have demonstrated that cytokine stimulation of hepatocytes coordinately and reciprocally regulates critical HDL apolipoproteins, increasing SAA and repressing both apoA-I and PON-1 expression. These changes were observed in 3 murine hepatocyte cell lines, in primary cultures of mouse hepatocytes, and in mouse liver in vivo after LPS injection. These cytokine-induced changes all were reversed by decreasing NF-κB activation or by PPARα agonists. Moreover, in the absence of the PPARα gene, isolated primary murine hepatocytes displayed a pattern of expression of these 3 HDL apolipoproteins similar to that seen after inflammatory stimuli.

Migita et al showed that stimulation of SAA by IL-1β in primary human hepatocytes was in large part related to activation of MAP kinase rather than NF-κB. Although these data differ from ours, their experiments were performed in human liver cells, in which no SAA response was observed to either TNF-α or IL-6, which is different to what we observed in murine hepatocytes. Moreover, in our experiments using a mixture of cytokines (IL-1β, TNF-α, and IL-6), the IL-1β effect represented only a small percentage of the response to the cytokine mixture used in AML-12 cells (supplemental Figure I). Although it is conceivable that MAP-kinase activation is playing a minor role in our system, which we are unable to detect by the use of MEK inhibitors, our findings using both chemical inhibitors and molecular approaches in vitro and our in vivo studies in mice indicate a critical role for NF-κB in the SAA response to cytokines. This reciprocal regulation of SAA versus apoA-I and PON-1 appears to involve an interaction between PPARαs and NF-κB. Although the apoA-I and PON-1 promoters do not have NF-κB sites, NF-κB inactivation has been shown to reverse LPS-induced suppression of apoA-I expression in a human hepatoma cell line. Moreover, a selective PPARα inhibitor blocked the increased apoA-I expression seen after overexpressing an IκB super-repressor, suggesting that NF-κB repression induces apoA-I expression through a PPARα-mediated mechanism. Our data indicate a similar effect for PON-1 and reciprocal finding for SAA.

Our findings extend insight into the transcriptional regulation of HDL apolipoprotein through PPARαs and NF-κB. Suppression of NF-κB activation with either chemical inhibitors or molecular approaches partially reversed the cytokine effects on the expression of SAA, which has NF-κB sites in its promoter, and on the expression of apoA-I and PON-1, which do not. We also demonstrate that the PPARα effects seen after LPS stimulation also apply to inflammatory cytokines, endogenous stimuli thought to promote atherosclerosis in vivo. We show that these effects extend to reciprocal and coordinated effects on PON-1 and SAA, and that all these effects require the genetic presence of PPARα. The tight, consistent, and coordinated reciprocal downregulation of apoA-I and PON-1 versus SAA upregulation during inflammation argues for a common mechanism involving cross-talk between PPARα and NF-κB. Although SAA does not have a PPRE in its promoter, its expression was increased in the basal state in hepatocytes from PPARα-deficient mice and the cytokine-induced expression of SAA was reduced by exposure of Hepa 1–6, AML12, and NMH hepatocytes to 2 different PPARα agonists. We plan to examine the effect of these transcription factors on the promoter activities of SAA, apoA-I, and PON-1 in future studies.

There are several possible mechanisms by which inflammation could affect both NF-κB and PPARα transcriptional activity. One mechanism would be regulation of the expression level of PPARα by cytokines. Others have shown that inflammation can repress PPARα expression, although we did not find a difference in the amount of PPARα protein. Another mechanism would be through direct interaction between PPARα and NF-κB. This will be the subject of future studies.
cross-talk between NF-κB and PPARα is markedly increased in PPARα-deficient mice. Consistent with findings of Staels,26-45 we also failed to show a consistent effect of fibrates on apoA-I expression in the absence of cytokine stimulation. It is possible that the effect of these agents on apoA-I expression is occurring indirectly through a PPARα-independent mechanism. However, the findings in hepatocytes from the PPARα-deficient mice strongly support some involvement of this nuclear receptor in the regulation of both SAA and apoA-I expression, even if indirect. Human apoA-I expression is known to be PPARα-regulated.25,26 Our results in the PPARα-deficient animals suggest that PPARα does affect the expression of mouse apoA-I, and that PPARα activation can amitigate NF-κB activation by inflammatory stimuli.

In vitro studies suggest that SAA can displace apoA-I from HDL particles.4,23 Our findings suggest an alternate explanation for the reduction in HDL levels seen during inflammation. Reduced expression of apoA-I by hepatocytes, the primary site of synthesis of HDL, with simultaneous increased SAA expression, would result in the secretion of HDL particles of altered composition. The simultaneous reduction in PON-1 expression would also contribute to alterations in HDL structure during inflammation, with likely functional consequences.

Inflammatory HDL has been shown to have a reduced capacity to inhibit LDL oxidation and the adhesion of monocytes to endothelial cells than control HDL,20,22 which has in part been attributed to the well-documented reduction in the PON-1 content of HDL that occurs during inflammation.21,22 Thus, PON-1 has been regarded as being atheroprotective, a hypothesis reinforced by the increased atherosclerosis that occurs in the PON-1-deficient mouse.46 The major atheroprotective apolipoprotein in HDL has been thought to be apoA-I, which plays a critical role in reverse cholesterol transport.49 ApoA-I transgenic mice are decreased atherosclerosis seen in the PON-1 transgenic mouse.50 The major atheroprotective lipoprotein in HDL has been thought to be apoA-I, which plays a critical role in reverse cholesterol transport.49 ApoA-I transgenic mice are protected against atherosclerosis, whereas atherosclerotic lesions are increased in apoA-I-deficient mice.51 Thus, inflammation-mediated reduction in the apoA-I content of HDL would be predicted to reduce the atheroprotective effect of HDL.

The role of SAA in atherogenesis remains unclear. Despite epidemiological associations between circulating SAA levels and cardiovascular risk in humans,1,2 there is no definitive evidence implicating SAA as having a direct role in atherogenesis or cardiovascular disease. However, we recently demonstrated that diet-induced increases in SAA levels associated with an increase in atherosclerosis in LDL receptor–deficient mice, independent of changes in plasma lipids and lipoproteins. Additionally, we showed that HDL binding to vascular proteoglycans in vitro was proportional to the SAA content of the lipoprotein and that SAA, apoA-I, and proteoglycans colocalized in lesions in these mice.6 These

Our data indicate an important role for PPARα in both limiting inflammation under basal conditions as well as countering proinflammatory stimuli. The elevated SAA and suppressed apoA-I and PON-1 levels in the unstimulated state in the absence of PPARα indicate that the cells were in an inflammatory state, implicating PPARα as a “brake” that limits inflammation even in the basal state. Further evidence for a role for PPARα in suppressing basal levels of inflammation comes from the observation that endothelial cell expression of adhesion molecules, an indicator of endothelial inflammation, is markedly increased in PPARα-deficient mice. ApoA-I–deficient mice also have prolonged responses to inflammatory stimuli.47 The major atheroprotective apolipoprotein in HDL has been thought to be apoA-I, which plays a critical role in reverse cholesterol transport.49 ApoA-I transgenic mice are protected against atherosclerosis, whereas atherosclerotic lesions are increased in apoA-I–deficient mice.51 Thus, inflammation-mediated reduction in the apoA-I content of HDL would be predicted to reduce the atheroprotective effect of HDL.

The observation that cytokines suppressed apoA-I expression by PPARα agonists in murine Hepa 1–6 cells was somewhat unexpected because the PPRE in rodent apoA-I has been reported to be mutated and nonresponsive to PPARα agonists.26 Nonetheless, experiments in 3 different murine hepatocyte cell lines using 2 different PPARα agonists, WY-14643 and fenofibrate, yielded similar results. Moreover, the changes in apoA-I expression was absent in hepatocytes from PPARα-deficient mice. 

Figure 5. PPARα agonists block the alterations of SAA, apoA-I, and PON-1 induced by cytokines. Hepa 1–6 and AML12 cells were treated with the PPARα ligands, WY-14643 (20 to 100 μM) and/or a mixture of cytokines. A, After incubation for 72 hours, culture media were analyzed by immunoblot. The intensity of the apoA-I bands (lower panel) was measured and plotted; the band intensity from untreated cells was set at 100%. Values shown are means ± SD. B, Total RNA after 24-hour exposure was analyzed by Northern blot.
A th of inflammatory cytokines, including TNF-α, IL-1, and IL-6, all at 10 ng/mL) for 24 hours. Nuclear extracts (10 μg) were analyzed by EMSA using 100 ng of radiolabeled NF-κB oligonucleotide. Specificity was determined by the addition of unlabeled NF-κB (cold probe) or mutant NF-κB oligonucleotide before the addition of the labeled probe. WY-14643 inhibited cytokine-induced activation of NF-κB in PPARα+/− but not in PPARα−/− hepatocytes.

In summary, the present findings suggest a novel mechanism through which inflammatory HDL apolipoproteins (apoA-I and PON-1) and the inflammatory and potentially atherogenic HDL apolipoprotein, SAA, are reciprocally regulated by inflammation. We propose a model whereby these reciprocal changes during cytokine-mediated inflammation are regulated by an interaction between PPARα and NF-κB, inducing counter-regulatory transcriptional responses through changes in expression of their target genes. Moreover, our findings provide further evidence for PPARα expression exerting a chronic “braking” effect on inflammation, which can be reversed by inflammatory cytokines or the absence of PPARα itself.

Sources of Funding
This work was supported by grants from the National Institutes of Health (HL30086, HL18645, HL079117, HL071745, HL048743, CA23226, and CA074131) and a grant from the Donald W. Reynolds Foundation.

Disclosure(s)
None.

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Arterioscler Thromb Vasc Biol. 2006;26:1806-1813; originally published online May 18, 2006; doi: 10.1161/01.ATV.0000227472.70734.ad
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL FIGURE LEGENDS

Figure I. Cytokines up-regulate SAA while simultaneously down-regulating apoA-I and PON-1 in hepatocytes. AML12 and NMH cells were treated with a mixture of cytokines (10 ng/ml TNF-α, 10 ng/ml IL-1, 10 ng/ml IL-6), or no additions. Total RNA was extracted and subjected to Northern blot analysis using SAA, apoA-I and PON-1 cDNAs as probes. A cDNA encoding GAPDH was used as a control for loading equivalence of RNA.

Figure II. Synergistic effect on SAA, apoA-I and PON-1 expression by combinations of cytokines. AML12 cells were treated with each cytokine alone or with a combination of cytokines (10 ng/ml TNF-α, 10 ng/ml IL-1, 10 ng/ml IL-6). Total RNA was extracted and was quantified by multiplex real-time RT-PCR using SAA1, apoA-I and PON-1 specific primers and probes, normalized to GAPDH. Values shown means±SD.

Figure III. LPS does not directly affect the expression levels of SAA and apoA-I. AML12 cells were treated with the mixture of cytokines used previously or with LPS (500ng/ml, 1μg/ml, 2μg/ml) added directly to the cells for 24 hr. Total RNA was extracted and analyzed by multiplex real-time RT-PCR using SAA1 and apoA-I as described in Figure II.

Figure IV. A mixture of cytokines does not change the expression levels of the nuclear transcription factors, NFκB and PPARα. AML12 cells were treated with a mixture of cytokines for 24 hr. Cell lysate was analyzed by immunoblot using specific antibodies against NFκB and PPARα.
Figure I
Figure II
Figure III
Figure IV

Cytokines

NFκB p65

PPARα