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Original Research Article

Differential regulation of peroxisome proliferator activated receptor isoforms in the macrophage J774.2 cell line by cytokines

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Abstract

The regulation of the peroxisome proliferator-activated receptor (PPAR) isoforms in macrophages by cytokines is of potentially crucial importance in the pathogenesis of atherosclerosis. However, the precise mechanisms by which different cytokines modulate the expression of macrophage PPAR isoforms activity are still poorly understood. In the present study, we evaluated the action of four cytokines on the expression of PPAR isoforms mRNA, protein and functional PPAR-DNA binding activity in the murine macrophage J774.2 cell line, a widely used model system for atherosclerosis. Exposure of the cells to tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) for 24h; produced a dose-dependent reduction of PPAR alpha and gamma isoforms (PPARα and PPARγ) and a dose-dependent increase of the PPAR beta/delta isoforms (PPARβ/δ) mRNA and protein expression. In contrast, interleukin-1beta (IL-1β) produced a dose-dependent increase of PPARα and PPARγ and a dose-dependent decrease in PPARβ/δ mRNA and protein expression. However, IL-1α has no effect on all isoforms of PPAR mRNA and protein expression. Electrophoretic mobility shift assay (EMSA) showed a close correlation between the expression of the PPAR mRNA, protein and the functional PPAR-DNA binding activity. Incubation of nuclear extracts with anti-PPAR antibodies in super-shift assay demonstrated the participation of all the three PPAR isoforms in the binding to the peroxisome proliferator response elements (PPRE). These results indicate that TNFα, IFNγ, IL-1α and IL-1β are important regulators of macrophage PPAR gene and protein expression which affect its DNA binding activities. Thus, this study provides novel insights in to the potential mechanisms that may be responsible for the mediator specific regulation of macrophage gene expression through the PPAR isoforms, indicating a possible physiological and potential role for this transcription factor in modulating arterial lipid metabolism and inflammatory response associated with atherosclerosis.

Keywords: nuclear receptors; atherosclerosis; acute phase response; macrophage; pro-inflammatory cytokines

1. Introduction

Atherosclerosis is the underlying cause of coronary artery disease which is the leading cause of morbidity and mortality worldwide [1]. Despite many advances in cardiology, atherosclerosis remains a major medical problem which results in disastrous clinical consequences, such as ischemia of the heart, brain or extremities infarction [2-4]. As a long-term chronic cardiovascular disease, atherosclerotic lesions develop as early as in teenagers and young adults, which progressively evolve over decades before manifesting into a clinical disorder [5]. An early event in the development of atherosclerosis is the accumulation of lipid-loaded, macrophage-derived foam cells, which participate in the initiation, progression and clinical complications of the disease [5, 6].

A large number of cytokines, small intercellular regulatory proteins, are well known to be secreted in great amounts by cells participating in atherogenesis. Indeed, in the early stages of atherosclerosis, pro-inflammatory cytokines such as IL-6, IL-1α, IL-1β, TNFα, IFNγ, regulate many critical cellular functions such as cell recruitment and migration of macrophages to the vascular wall [4, 7, 8]. Progressive accumulation of macrophages and their uptake of modified lipoprotein in the arterial wall, ultimately, lead to the development of atherosclerotic lesions [9, 10].

A family of transcription factors known as peroxisome proliferator activated receptors (PPARs) has been demonstrated to plays a central role in regulating metabolic risk factors for cardiovascular disease. The discovery of PPARs was highly significant and since then, many studies worldwide are being carried out on PPAR's three isoforms (α, β/δ and γ) involved in the control of vascular inflammation related to atherosclerosis [11].

Indeed, PPARα isoform, which is known to play an important role in the metabolism of fatty acids, lipids and lipoprotein, has also been implicated in atherogenic and inflammatory processes [1, 12-14]. Also, other data have indicated that PPARα activators exert anti-inflammatory activities in cardiovascular disease [6, 15]**.** Therefore, the PPAR isoforms play important roles in the development of atherosclerosis by interfering with proatherogenic processes at different levels. PPARα exerts beneficial effects on atherosclerosis by exchanging plasma lipid and lipoprotein profiles toward less atherogenic levels. PPARα also interferes with the development of atherosclerosis by inhibiting inflammatory responses at the level of vascular wall. In contrast with other PPAR isoforms, PPARβ/δ has not been very well studied but may play an important role in atherosclerosis development by acting in the modulation of vascular smooth muscle cell (VSMC) proliferation [16]. Indeed, Zhang *et al* (2002) showed that PPAR β/δ is expressed in VSMCs and is upregulated during vascular lesion formation in the neointima [17]. Moreover, platelet-derived growth factor (PDGF) induces PPARβ/δ overexpression in VSMCs which, in turn, promotes the proliferation of confluent cells. PPARγ, on the other hand, is expressed abundantly in macrophage foam cells of human atherosclerotic lesions and has found to regulate endothelial function and vessel wall inflammation [18, 19].

Many known factors, such as the OxLDL, macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) were known to be present in the atherosclerotic plaque. These factors induced PPARγ expression in murine peritoneal macrophages *in vivo* [20]. In addition to that, PPARγ specific ligands, such as rosiglitazone, were found to strongly inhibit the development of atherosclerosis in male LDL- receptor deficient mice [21]. The PPARγ activator troglitazone inhibits smooth muscle cell proliferation and decreases the intima and media thickness in human carotid arteries [22].

More recently, the effects of cytokines on macrophage PPARs expression has received a substantial interest due to their involvement during initiation and progression of atherosclerosis. For instance, it has been shown that IL-4 induces PPARγ expression in macrophages [20, 23]. Whereas TNFα, IL-1β and IL-6 diminish the expression of PPARγ in rat adipocytes [24]. However, studies carried out on the roles of PPARs in macrophages and their regulations by cytokines in the progression of atherosclerosis were more limited.

Because cytokinenin are known to modulate the progression of atherosclerosis by regulating the function of various cells present in the lesion, their action on macrophage PPARs may, therefore be equally important in the pathogenesis of atherosclerosis.

Therefore, in this present study, we explore the effects of IL-1α, IL-1α, TNFα, and IFNγ on PPAR isoforms activity, steady state mRNA levels, protein content and PPAR-DNA binding activities in the murine macrophage J774.2 cell line in the modulation of atherosclerosis.

2. Materials and Methods

2.1 Materials

The J774.2 cell line was obtained from the European Collection of Animal Cell Cultures (ECACC). The cytokines TNFα, IFNγ, IL-1γ and IL-1α were obtained from National Institute for Biological Standards and Control, UK. All the cell culture reagents were purchased from Gibco BRL (Paisley, UK).

2.2 Cell culture

J774.2 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM). The medium was supplemented with 2mM of L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin, with 10% (v/v) heat-inactivated (30min, 56°C) fetal calf serum (FCS). The cells were maintained in a humid incubator of 5% (v/v) CO_2 at 37°C. The cell culture medium was replaced every three days. Before incubation in the presence of cytokines, the cells were pre-incubated for 4h in medium containing reduced FCS [0.5% (v/v)]. The medium was then removed and replaced with fresh medium [with 0.5% (v/v) FCS] containing individual cytokines and incubated for 24h. For untreated samples, fresh medium plus 0.5% (v/v) FCS, in the absence of cytokines, was used.

2.3 Primer design

Nucleotide sequences of murine PPARα, PPARβ/δ, PPARγ and β-actin cDNA (Accession No: NM_011144, NM_011145, NM_011146 and NM_007393, respectively) were obtained from the website of National Centre of Biotechnology Information, USA. (http://www.ncbi.nlm.nih.gov/).

2.4 RNA isolation and RT-PCR analysis

Total RNA was isolated from treated and untreated samples of cells using the Tri-Reagent LS (Molecular Research Center) according to the manufacturer's instructions. The concentration and purity of the isolated RNA was determined by measuring the OD at 260nm and 280nm using GeneQuant Calculator (Pharmacia). Each isolated RNA sample was size-fractionated on denaturing 1% (w/v) agarose formaldehyde gels containing. DNase treatment was performed by subjecting 2µg of RNA to 10U of RNase-Free DNase (Promega). Synthesis of first strand cDNA was carried out using total cellular RNA (2µg).

Then, a PCR was performed using 2.5U of Taq polymerase (Promega). The PCR products were then size fractioned on 1.2% (w/v) agarose gels, visualised using a UV transilluminator, photographed using GeneSnap software and subsequently analysed using Gene Tools analysis software on GENE GENIUS gel documentation system (Syngene).

2.5 Western blot analysis

Total cellular protein was isolated using Tri-Reagent LS (Molecular Research Center), according to the manufacturer's instructions. The final protein pellet was then dissolved in 1% (w/v) SDS. The concentration of total cellular protein was determined using Bio-Rad DC Protein Assay Kit (Bio-Rad). A total of 30µg of protein was used in SDS-PAGE in order to determine the levels of protein content for PPARα, PPARβ/δ, PPARγ and β-actin, respectively. Subsequently, proteins were transferred to a polyvinylidene difluoride membrane (Milipore) and incubated with blocking solution [1X PBS containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20] for 1 hr at room temperature with shaking. The membrane was washed three times for 10 min each, in washing solution [1X PBS and 0.1% (v/v) Tween-20] and incubated with primary antibodies (rabbit anti-mouse PPARα, PPARβ/δ, PPARγ and β-actin) which was diluted 1/1000 in 1X PBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20, for 1 hr at room temperature. The membrane was then washed and immersed in secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) diluted 1/2000 in 1X PBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20. Detection of membrane bound antigen-antibody complexes was carried out as described in the instructions supplied with the ECL kit (GE Healthcare) and Fuji Medical X-ray film. WestviewTM Western Size Marker (Mbiotech Inc. Songpagu, Seoul) and immunoreactive proteins were visualized on X-ray film.

2.6 Electrophoretic Mobility Shift Assay (EMSA) analysis and antibody supershift assay

Nuclear extracts used in EMSA were extracted using NE-PER Nuclear Cytoplasmic Extraction Reagents (Pierce). The sequences of the oligonucleotides containing the PPRE bindingsite were as follows:

5'-AACTAGGTCAAAGGTCATC-3' and

5'-AGGGGATGACCTTTGACCTAG-3'.

The oligonucleotides were labeled using biotin 3'-End DNA Labelling kit (Pierce) according to the manufacturer's instructions. For binding reactions, 20 µg of nuclear extracts were mixed together with labeled oligonuclotides using LightShift Chemiluminiscence EMSA kit (Pierce) according to manufacturer's instructions.

DNA-protein complexes were then resolved by electrophoresis using 6% (w/v) non-denaturing polyacrylamide gels (29:1, acrylamide: bisacrylamide). DNA-protein complexes were transferred to nylon membrane (Hybond N+) and subsequently, detection of the biotin- labeled DNA on the membrane was carried out using Fuji Medical X-ray film. For antibody super-shift assays; 1 µg of PPARα, PPARβ/δ or PPARγ antibody (Cell Signaling Technology) was mixed with the binding reactions and the mixture was incubated on ice for 20 min before the labeled oligonucleotides were added.

3. Results

3.1 The effects of cytokines on PPAR isoforms mRNA levels of macrophages

The effects of individual cytokines on PPAR isoforms gene expression in J774.2 macrophages were shown in Figure 1. The RT-PCR analysis showed a differential expression profile for PPARα, β/δ and γ in J774.2 cells stimulated with various concentrations of cytokines for 24h. The expression of PPARα was highly expressed in the untreated (UNT) cells and decreased steadily in dose dependent manner upon exposure to TNFα and IFNγ reaching its lowest levels at 1000 U/ml (Figures 1A and B). By comparison, IFNγ was more effective in producing a reduction in PPARα gene expression level than TNFα at 500 U/ml and 1000 U/ml (reduction to 26% and 38% compared to untreated cells for IFNγ, and 16% and 22% for TNFα respectively). By contrast, there was a dose dependent increase of 32% compared to the untreated in PPARα mRNA expression when the cells were stimulated with 0-500U/ml IL-1β (Figure 1C). However, increasing the concentration of IL-1β to 1000U/ml did not produce any significant change in PPARα mRNA level as compared to cells treated with 500U/ml. In contrast to PPARα, the PPARβ/δ expression level was dose-dependently increased following treatment of J774.2 cells with TNFα and IFNγ, reaching their maximum levels at 1000U/ml (30% and 39% increase, respectively, compared to untreated cells) [Figure 1A and B]. On the other hand, there was a steady dose-dependent reduction of 28% was observed in PPAR β/δ expression level when the cells were treated with 1000U/ml IL-1β for 24h (Figure 1C). Interestingly, a similar dose- dependent reduction of PPARγ gene expression pattern was observed when the cells were incubated with various cytokine concentrations of TNFα and IFNγ as demonstrated in PPARα mRNA suppression (Figure 1A and B). However, the level of maximal suppression exerted by TNFα and IFNγ at 1000U/ml was higher on PPARγ (86% and 42%, respectively) as compared to PPARα mRNA expression (22% and 38%, respectively). The decrease in PPARγ expression level, relative to the untreated cells, produced by 1000 U/ml of TNFα was about two-fold greater than that observed with IFNγ. PPARγ expression level produced by IFNγ was 42% (Figure 1B) as compared to 86% decrease as observed with TNFα (Figure 1A). When the J774.2 cells were stimulated with IL-1β, PPARγ expression level exhibited a similar dose-dependent increase expression as PPARα (Figure 1C). However, the increase was only marginally about 19% compared to the untreated at 1000U/ml. By contrast to the cytokines previously discussed as above (TNFα, IFNγ, and IL-1β), IL-1α, however, had no effects on the expression of any of the PPAR isoforms through the 24h period of incubation with the various concentrations of individual cytokine (Figure 1D). Therefore, these results demonstrated that both TNFα and IFNγ exhibited almost similar

pattern of differential regulation of PPAR isoforms mRNA expression in dose dependent manner (decrease in PPARα and PPARγ but an increase in PPARβ/δ gene expression). By contrast, IL-1β produced an opposite differential regulation of PPAR isoforms gene expression as compared to TNFα and IFNγ (increase in PPARα and PPARγ and decrease in PPARβ/δ gene expression) whereas, IL-1α showed no significant effects on the expression of all three PPAR isoforms at mRNA levels.

3.2 The effects of cytokines on PPAR isoforms protein content of macrophages

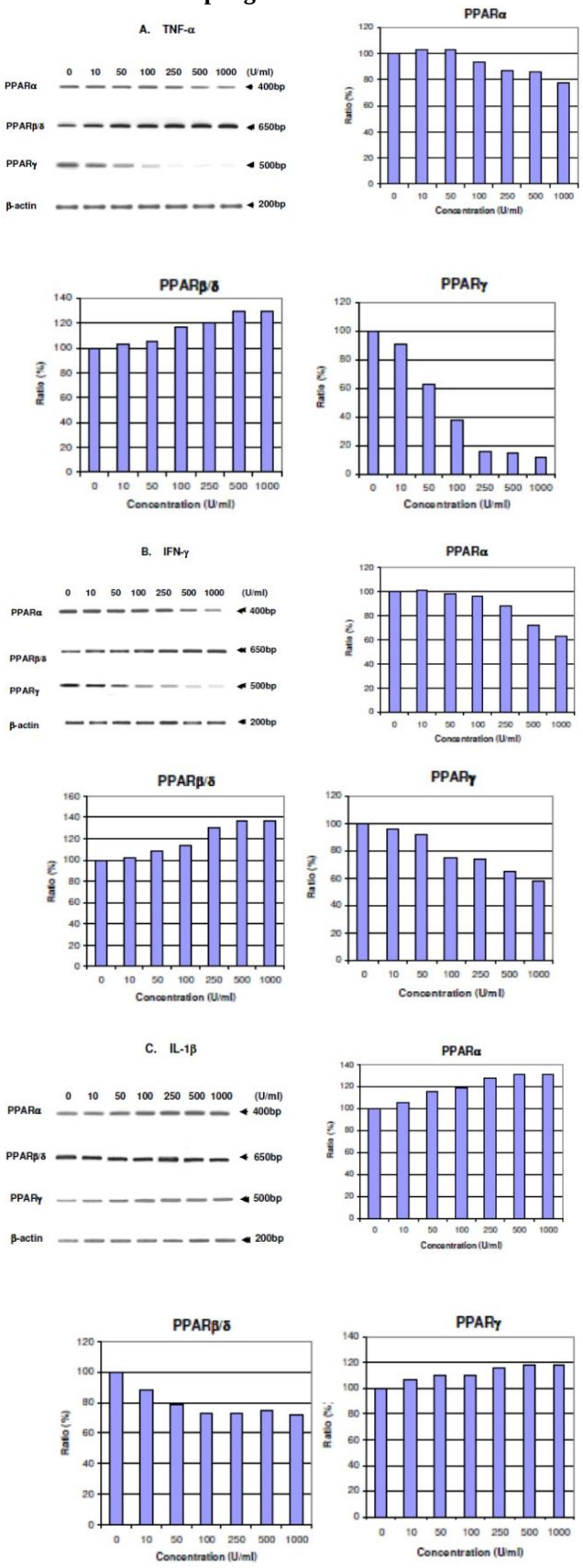
In order to determine whether the changes observed in PPAR isoforms mRNA expression in J774.2 cells treated with cytokines (concentration of 1000U/ml) were also observed by the corresponding changes at the protein level, Western Blot analysis was carried out. As shown in Figure 2, there was a differential protein expression pattern for PPAR isoforms in J774.2 cells treated with various cytokines. The levels at maximal concentration (1000U/ml) closely followed the corresponding changes in the pattern of mRNA expression (Figure 1, panel A- D). Cells stimulated with TNFα and IFNγ produced a decrease in the PPARα and PPARα, but an increase in PPARβ/δ protein content. A similar pattern of expression between the mRNA and the protein expression of PPARδ, PPARβ/δ and PPARγ, was also observed when the cells were treated with IL-1β. In this case, it was demonstrated that PPARα and PPARγ protein expressions were increased but PPARβ/δ protein expression was reduced in treated cells. As shown in Figure 2, treatment of cells with IL-1α produced no significant changes in the apparent PPAR isoforms protein content of the cells which was also observed at mRNA levels. Following GeneTools analysis of the Western Blots, the overall magnitude of decrease in the PPAR isoforms protein content observed following incubation in the presence of each of the cytokines at 1000U/ml was between 40-45% (PPARα), 20% (PPARβ/δ) and 50-70% (PPARδ), almost similar to the corresponding reduction in the PPAR isoforms mRNA expression levels [22-38% (PPARα), 28% (PPARβ/δ) and 42-86% (PPARγ)] compared to the untreated cells. The similar pattern of magnitude of protein induction was found to be 28% (PPARα), 20-30% (PPARβ/δ) and 15% (PPARγ) corresponding to the induction in the PPAR isoforms mRNA expression levels [32% (PPARα), 30-39% (PPARβ/δ) and 19% (PPARγ)]. Thus, these data suggest that the differential expression of PPARs protein content by TNFα, IFNγ, IL-1α and IL-1 β was due, mainly, to the corresponding changes in the mRNA expression level.

3.3 The effects of cytokines on PPAR–DNA activity of macrophages

In order to identify whether these changes in PPAR isoforms gene and protein expressions were also followed by corresponding changes in the DNA binding activity of PPARs, an Electrophoretic Mobility Shift Assay (EMSA) was carried out using nuclear extracts prepared from J774.2 macrophages incubated with individual cytokines at the maximal concentrations (1000U/ml). Figure 3A shows the outcome of these experimental series. At least three DNA- protein complexes, designated C1, C2 and C3 were apparent in EMSA using nuclear extracts from untreated cells. Complexes C2-C3 were the most prominent with C1 representing a minor component. None of these complexes was present when only the labeled oligonucleotides were used in the absence of nuclear extracts [(Lane FP), Figure 3A and B]. As shown in Figure 3A, treatment of the cells with TNFα resulted in a significant decrease in the binding of nuclear proteins to the PPRE oligonucleotide. However, there was only a slight decrease in PPAR binding activity in J774.2 cells treated with IFNγ. By contrast, PPAR-DNA binding activity was increased when the cells were treated with IL-1β but demonstrated no changes when treated with IL-1α against the control. These results obtained from EMSA demonstrated that PPAR binding activity closely followed the patterns of PPAR isoforms mRNA and protein expressions in J774.2 cells treated with maximal concentration of cytokines at 1000U/ml. Also, we employed the antibody-supershift assays (Figure 3B) to identify the nature of the PPAR isoforms which participated in the formation of the complexes. Figure 3B shows the outcome of the experiments using untreated nuclear extracts, antibodies against PPARα, PPARβ/δ and PPARγ. In common with previous studies, inclusion of antibody against PPAR isoforms resulted in the formation of slower, antibody-protein-DNA 'super-shifted' complexes of higher molecular weight [25].

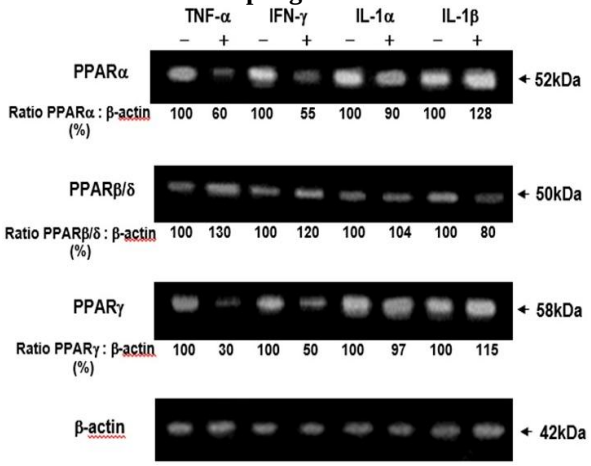
Furthermore, the formation of complexes C1 and C3 were reduced by antibodies against PPAR isoforms [26]. Such a decrease in the binding activity to the PPRE sequence and the induction of the super-shifted bands upon incubation with antibody clearly suggest that all PPAR isoforms participated in the DNA-protein interactions.

Figure 1: PCR analysis showing the effects of cytokines on PPAR isoforms mRNA expression in murine macrophage J774.2 cell line



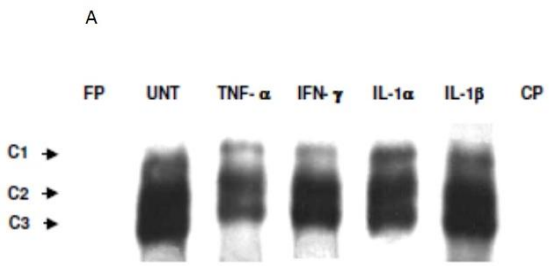
J774.2 cells were made quiescent by serum starvation [0.5% (v/v) FCS] for 4 h and then stimulated for 24 hr with different concentrations of (A) TNFα, (B) IFNγ, (C) IL-1α or (D) IL-1β, as shown. β-actin was used as an internal control for the integrity and equal amount of cDNA used in each PCR reaction. Graphical PCR analysis representations showing the effects of cytokines on PPAR isoforms mRNA levels in murine macrophage J774.2 cell line. The graphs represent a mean of two independent experimental series that each gave similar patterns. The PPAR: β-actin ratio in untreated cells has been assigned as 100%, with the ratio for the remaining samples being represented relative to this control value. The data shown from the expression pattern is representative of two independent experimental series.

Figure 2: Western Blot analysis showing the effects of cytokines on PPAR isoforms protein expressions in murine macrophage J774.2 cell line



Samples of total cellular proteins (30µg) was determined by protein assay and size- fractionated in 10% (v/v) SDS-PAGE, blotted onto nitrocellulose and probed with rabbit anti- mouse PPARα, PPARβ/δ, PPARγ and β-actin as indicated. β-actin was used as an internal control for the integrity and equal amount of total cellular protein used in each Western Blot analysis. The PPAR isoforms: β-actin ratio in untreated cells has been assigned as 100%, with the ratio for the remaining cell samples being represented relative to this control value (indicated by numbers in the figure). The data shown is representative of two independent experimental series, each of which produced the same pattern of changes.

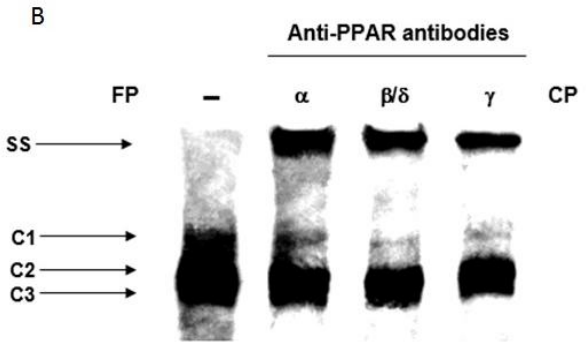
Figure 3: (A) EMSA supershift analysis showing PPRE binding activity of nuclear extract in murine macrophage J774.2 cell line incubated in the presence of individual cytokine.



J774.2 cells were either untreated (UNT) or incubated for 24 hr with 1000U/ml TNF- α, IFN-γ, IL-1α or

IL-1β as indicated. Nuclear extracts (15µg) were then used for EMSA. Three DNA-protein complexes, denoted as C1, C2 and C3 were detected and are indicated by labelled arrows. The free probe was allowed to migrate off the gel. FP (free probe) represents biotin-labeled PPRE oligonucleotide alone and CP (cold probe) represents competition assay using 100-fold molar excess of unlabelled PPRE oligonucleotide. The data shown is representative of two independent experimental series.

Figure 3: (B) EMSA antibody supershift analysis showing the nature of PPAR isoforms involved in DNA protein interaction in murine macrophage J774.2 cell line.



EMSA antibody supershift analysis was used to determine all PPAR isoforms present in shifted complexes and identify the nature of the PPARs proteins which participated in the formation of the complexes. Nuclear extracts (15µg) from J774.2 cells were used for EMSA in the absence of antibody (-) or presence of anti-PPAR antibodies (PPARα, β/δ, γ). (α) represents nuclear extract + anti-PPARα antibody; (β/δ) represents nuclear extract + anti- PPARβ/δ antibody and (γ) represents nuclear extract + anti-PPARγ antibody. (SS) denotes the super-shifted complexes. FP (free probe) represents biotin-labeled PPRE oligonucleotide alone and CP (cold probe) represents competition assay using 100-fold molar excess of unlabelled PPRE oligonucleotide. C represents pattern of DNA-protein complexes (C1, C2 and C3).

4. Discussion

This study demonstrated that exposure of the cells to TNFα and IFNγ for 24h, produced a dose dependent reduction of PPARα and PPARγ and a dose-dependent increase of PPARβ/δ mRNA and protein expression. In contrast, IL-1β produced a dose-dependent increase of PPARα and PPARγ and a dose-dependent decrease in PPARβ/δ mRNA and protein expression. However, IL-1α has no effects on all isoforms of PPAR mRNA and protein expression. EMSA showed a close correlation between the expression of the PPAR mRNA, protein and the functional PPAR DNA binding activity. In addition, antibody super-shift assay demonstrated the participation of interactions between all the three PPAR isoforms in the binding to PPRE.

These results demonstrate for the first time that TNFα and IFNγ dose-dependently regulate PPAR isoforms expression in murine macrophage J774.2 cells. The decreased in the pattern of PPARα gene expression following stimulation with TNFα has also been investigated in various cell types [27]. Using a novel dot blot RNase protection assay and densitometric analysis, mRNA and protein expression of PPARα in male *Spraque-Dawley* rat liver was significantly reduced about 30% compared to the control after 16h administration of 25 µg TNFα [27]. In addition to the inhibitory effect on PPARα, TNFα also increased the expression of transcription factor c-jun [28]. Tengku Muhammad *et al*., (2000b) demonstrated that c-Jun expression was increased in a biphasic manner when murine macrophage J774.2 cells were treated with TNFα with peak expression occurred at 30 min and 16h [29]. Both c-Jun and PPARα were known to mutually inhibit each other in transactivation functions [30]. Thus, it is tempting to speculate that TNFα inhibitory effect on PPARα expression levels may be mediated in the activation of c-Jun expression. The direct reduction of PPARα expression level and the induction of c-Jun expression which may inhibit the PPARα function may have an additive effect in the diminution of peroxisomal β-oxidation, which plays an important role for the degradation of very long chain and polyunsaturated fatty acids [13]. A decrease in peroxisomal β-oxidation that involves in degradation of prostaglandins and leukotrines may contribute to the intensification of inflammation-induced effects by TNFα as PPARα is known to play a central role in inflammation control of atherosclerosis [27, 31].

By contrast to the decrease of PPARα gene expression, the expression of PPARβ/δ was increased by approximately 30% compared to the untreated cells following stimulation of macrophage J774.2 cells with TNFα. Studies have demonstrated a similar induction of other transcription factors such as AP-1 members, C/EBPβ and C/EBPδ mRNA expression in various cells/ tissues by this cytokine [29, 32]. For example, in cultures of rat hepatocytes, the level of C/EBPβ and C/EBPδ mRNA was elevated by stimulation with 1000 U/ml TNFα, with maximum expression being observed at 3-4h [33, 34].

The most intriguing finding of this study was that J774.2 cells stimulated with IFNγ revealed a similar pattern in PPARα, PPARβ/δ and PPARγ expression as compared to TNFα treatment. Similar to TNFα, IFNγ is known primarily for its roles in immunological responses and has the ability to affect the expression of many adipocyte transcription factors, including PPARγ1 and -γ2, C/EBPα, C/EBPβ, SREBP-1, and STAT3 [35]. However, the most profound effect of IFNγ was on PPARγ expression [35]. Indeed, they demonstrated a 2h treatment of 100U/ml IFNγ over a 24h time-course treatment in murine 3T3-L1 adipocyte cell line resulted in a substantial loss of PPARγ mRNA which are in agreement with the effects of IFNγ on the J774.2 cell line observed from this study. We also

showed that IFNγ regulated PPARγ mRNA expression, by inhibiting the rate of transcription of PPARγ. Thus, it may seem reasonable to speculate that the development and the progression of atherosclerosis is modulated, at least, in part by cytokines such as IFNγ by limiting the tendency of macrophages to take on the characteristics of foam cells. Therefore, IFNγ plays a precise role as an atheroprotective mediator which executes anti-atherogenic effects in the atherosclerotic lesion [36]. The IFNγ has also been shown to regulate gene transcription factors, such as STAT family, via the tyrosine kinase pathway [35, 37, 38]. Upon IFNγ activation, STATs bind to and transactivate sites (GAS) present in the promoter region of target genes, and this may be responsible for the suppression of PPARγ [35, 39]. Therefore, it can be hypothesized that the STAT family may act as repressor of transcription of PPARα and PPARγ [15, 35]. However, the mechanisms of IFNγ downregulates the mRNAs for PPARα and PPARγ remains to be elucidated.

By contrast to the decrease of PPARα and PPARγ expression detailed above, the expression of PPARβ/δ was rapidly increased in a dose-dependent manner following stimulation with IFNγ. Several studies have also demonstrated a similar induction by IFNγ in other transcription factors such as c-Fos, c-Jun, Jun B, C/EBPβ and C/EBPδ mRNA expression [40] and macrophage inflammatory protein (MIP). Thus, IFNγ may have a pro-atherogenic effect and acts to modulate the expression of a number of genes implicated in cellular cholesterol homeostasis and foam cell formation of the necrosis core by inhibiting cholesterol efflux pathways [36, 41]. Thus, it is tempting to speculate that in this study, the increase expression of PPARβ/δ upon treatment with TNFα and IFNγ demonstrated the pro-atherogenic effect of PPARβ/δ as a powerful promoter of macrophage lipid accumulation in the arterial wall. Several studies have also implicated that PPARβ/δ expressed in vascular smooth muscle is up-regulated during vascular lesion formation by promoting the proliferation of confluent cells in the neointima [17, 42].

In contrast to TNFα and IFNγ, PPARα and PPARγ gene expression was increased, whereas PPARβ/δ was decreased following stimulation of J774.2 cells with IL-1β (Figure1A- C). A similar pattern of induction in PPARα and PPARγ gene expression was demonstrated in adipose tissue and skeletal muscle stimulated with insulin and glucocorticoids [43-45].

However, Bordji *et al* (2000) recently reported a reduction in PPARα and PPARγ gene expression following treatment by IL-1β in rat chondrocytes [46]. These authors demonstrated that stimulation of chondrocytes with IL-1β at 25 or 250 U/ml for 12h slightly decreased the expression of PPARα, whereas, the expression of PPARγ mRNA was significantly decreased by 60% and 70% respectively in a dose-dependent manner. Although this may seem contradictory to what has been reported here in this study, it must be pointed out that tissue/cell specific mechanisms may exist in the regulation of PPAR by cytokines. Firstly, both PPARα and PPARγ expression are differentially regulated in various cell/tissue types, such as macrophages, adipocytes and chondrocytes [47]. Secondly, the 12 hr of treatment with cytokines, which is the time used in work of Bordji *et al* (2000) and Suzawa *et al* (2003), is not enough to see a statistically significant and apparent pattern of PPARγ expression level, compared to 24h treatment [46-48]. Furthermore, most susceptible genes such as those coding for transcription factors were also induced in response IL-1 treatment, for example, NF-κB [49, 50] NF-Y [51], STAT [52, 53] and Oct-1 [54] which will direct or indirectly effect the expression of PPAR isoforms in several cellular pathways.

Unfortunately, previous studies with these cytokines on macrophage have been inconclusive, with either no effect or suppression of the PPAR expression being observed. For example, previous observation on the effects of TNFα on PPARγ were based on the use of a single dose cytokine on murine adipocytes 3T3-L1 (5ng/ml) and brown rat adipocytes (10nM) [55, 56]. Such discrepancies emphasized the major limitation of previous investigations on macrophage PPAR in that they have been unsystematic, incomplete and have not addressed the need to analyze the response of a consistent cellular system to wide range of mediator doses using a full range of available cytokines. Therefore, further studies are required to clarify to what extent transcriptional and posttranscriptional components are involved in differential regulation by cytokine treatments from mRNA expression to protein expression and beyond.

By using Western Blot analysis, it was demonstrated that TNFα, IFNγ and IL-1β also differentially regulated the PPAR isoforms protein expression in a manner almost similar to the corresponding change at the mRNA level. As expected, IL-1α, had no significant effect on PPAR isoforms protein expression in J774.2 cells (Figure 2). Thus, the differential expression of PPAR isoforms protein observed when the cells were stimulated with TNFα, IFNγ and IL-1β for 24h could be accounted for, mainly, by corresponding changes in the expression pattern in PPAR mRNA expression (Figure 1A-C). Thus, it can be concluded that the responses occur mainly at the level of mRNA metabolism by modulating PPAR mRNA transcription or stability. This general similar and parallel trend in both the expression pattern of individual PPAR proteins and their respective mRNAs, suggested that regulation of mRNA (i.e. gene transcription or mRNA stability) made a major contribution to the regulation of PPAR gene expression and protein content [26, 40, 57].

It was previously found that, IFNγ reduced PPARγ protein content through regulating a cellular event in inhibition of synthesis of PPARγ mRNA in 3T3-L1 adipocytes. A 24h treatment of IFNγ decreases both PPARγ mRNA and its protein expression [35]. As IFNγ has been

shown to affect fat metabolism and adipocyte gene expression in atherosclerosis, these data are, therefore, in agreement to the IFNγ mediated changes identified in the present study. In addition, using Western Blot analysis of isolated cytosolic and nuclear protein samples, Waite *et al.* (2001) further proved that majority of PPARγ was present in nucleus, and the amount of nuclear PPARγ protein substantially reduced after 6h treatment.

These results strongly suggest that changes in mRNA metabolism (i.e. gene transcription or mRNA stability) which, in turn, were also largely responsible in modulating the PPAR isoforms protein expression, were primarily responsible for the identified changes in the PPAR DNA binding activity following stimulation of the cells with cytokines. The changes in the pattern on the binding activity of nuclear PPAR isoforms to PPRE sequence of human LPL gene promoter have also been observed when murine macrophages were exposed to glucose [26]. According to their study, exposure of J774.2 cells to high glucose environment resulted in a significant increase in the expression of PPAR protein isoforms and their binding of nuclear proteins to the PPRE consensus. Based on the antibody supershift analysis, all PPAR isoforms displayed shifted bands, indicating that all three PPAR isoforms in the untreated nuclear extract of murine macrophage J774.2 cells participated in DNA-protein interactions. Thus, these PPAR isoforms appeared to be functional and active that binds to PPRE. This suggests that heterodimers of these proteins may play a key role in cytokine-mediated macrophage gene expression. PPAR isoforms may also play an important role, either directly or indirectly, in inhibiting or inducing the transcriptional activity of the target genes which contain PPRE in their promoter region in response to stimulation by cytokines.

We showed that the expression levels and DNA binding activity of macrophage PPAR isoforms are differently regulated by TNFα, IFNγ and IL-1β and is not significantly affected by IL-1α. Thus, it may seem reasonable to speculate that the development and the progression of atherosclerosis is modulated, at least, in part, by the local concentrations of cytokines such as TNFα, IFNγ and IL-1β in regulating macrophages to take on the characteristics of foam cells via modulating the expression or activity of PPAR isoforms.

This study has demonstrated that macrophage PPAR was differentially regulated by cytokines. The changes observed at PPAR mRNA levels were paralleled with PPAR protein expression and subsequently at the levels of DNA binding activity clearly indicate that mRNA metabolism was primarily responsible for the observed changes. Possible cell/tissue-specific mechanisms and pathways may exist for the modulation of PPARs gene expression by cytokines.

5. Conclusion

In conclusion, it has been demonstrated clearly that macrophage PPAR isoforms can be differentially regulated by TNFα, IFNγ, IL-1β and IL-1α cytokines, thus, exhibiting a complex process of cascade with numerous factors interacting with each other and their respective DNA binding sites to activate or inhibit the transcription of target genes which participates in atherosclerosis disease. The precise importance of these changes in the suppression or induction of the expression and activity of PPAR and subsequently PPAR regulated genes remains to be determined. Therefore, the present work has provided the basis for future study to further understand the molecular mechanisms and signal transduction pathways by cytokine signals, especially to elucidate the molecular mechanisms of signal transduction pathways taken by IFNγ to decrease the levels of murine macrophage PPAR gene expression, and define the *trans*-acting factor(s) activated by the identifying signal transduction pathways that mediate the IFN-γ-inhibitory effects on murine macrophage PPAR isoforms.

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