Leptin Increase Matrix Metalloproteinase-9 (MMP-9) Secretion By Chondrocytes-Induced IL-1β, Through PPAR-γ Suppression

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Abstract: Obesity is a major risk factor for osteoarthritis (OA). Adipose tissue is an active endocrine organ that secretes cytokines and other molecules, including leptin. MMP-9 is an enzyme that plays an important role in the destruction of cartilage. PPAR-γ is a transcription factor that plays a role in metabolism of chondrocytes. The aim of this study is to determine the effect of leptin in MMP-9 secretion as a degradative enzyme by chondrocytes, as well as the involvement of PPAR-γ in the process. This study used human chondrocytes cell line that induced with IL-1β and then exposed to leptin. Secretion of MMP-9 was measured by ELISA, while PPAR-γ expression was measured by RT-PCR. Leptin addition increased MMP-9 secretion a dose dependent manner. Addition of PPAR-γ agonist pioglitazone, decrease MMP-9 secretion significantly. Moreover, addition of low and intermediate dose of leptin could not decrease PPAR-γ expression, while addition of high dose of leptin decreased PPAR-γ expression significantly. There was a strong correlation between MMP-9 secretion increase and PPAR-γ expression decrease. These results suggest that leptin may have a role in the pathogenesis of OA by increasing the chondrocyte catabolic response, and this effect is mediated at least by PPAR-γ.

1. Introduction
Osteoarthritis (OA) is the most common chronic musculoskeletal abnormality and form of arthritis to be found (Fahmi, 2011). Osteoarthritis is an illness caused by multifactor which onset and progression are related to aging, obesity, trauma, and unstable joint (Mandelbaum and Waddell, 2005). Obesity on its own is the main risk factor for OA (Ouellette and Makowski, 2010).

Several studies have found positive correlations between obesity and the onset of OA. The risk of OA in obese people rise from 9% to 13% in each kilogram gained by them (Ouellette and Makowski, 2010). What occurs in obesity are increased collagen solidity, mechanical change of extracellular matrix, and decreased proteoglican synthesis, which lead to cartilage degradation (DeGroot, 2004). It has been long believed that obesity trigger OA due to the overloading mechanical weight on the joints, but more recent data has proven that other processes were taking place in obese people; as shown by OA occurrence in an obese individual. This led to a new paradigm that metabolic factors are also involved in the patophysiology of OA (Erlangga, 2012).

Adipose tissue, abundance in obese people, is an endocrine organ that actively secreting cytokine and other molecules. A group of molecules secreted by adipose tissue is adipokine, consisting leptin, adiponectin, resistin, and visfatin. Leptin is a cytokine-like hormone which level is high in obesity (Simopoulou et al, 2007). There is a direct correlation between leptin level in synovial fluid and Body Mass Index (Otero et al, 2005). Leptin may increase some catabolic factors in OA progression and joint cartilage destruction. Patients with OA have higher leptin plasma level than patients without OA (Dumond et al, 2003). Leptin may also induce PPAR-γ phosphorylation in serin 112/82 and lead to decreasing PPAR-γ expression (Kimata et al, 2010).

The pathophysiology basis of joint damage in OA is the imbalance in degradation speed of extracellular matrix by catabolic enzymes matrix metalloproteinase (MMP) compared to the inhibition of extracellular matrix degradation by MMP endogen inhibitor i. e. , tissue inhibitor of metalloproteinase (TIMP). Chondrocytes, as the main cell in the cartilage, in OA condition is functionally and structurally impaired in secreting matrix components (Fuerst et al, 2011). Chondrocytes is induced by cytokines such as Interleukin-1 (IL-1) and Tumor Necrosis Factor alpha (TNFα) produced by synovial cells or macrophage to produce catabolic components. Interleukin-1 is a potent pro-inflammation cytokine that may stimulate chondrocytes to re-synthesize IL-1 and other pro-inflammation cytokines like IL-6, and synthesize various MMP degradative enzymes. Several MMPs known to play a role in OA pathogenesis are MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 (Gomez et al, 2009).

Recently, the role of MMP-9 in OA pathogenesis has attracted the interest of many researchers. In OA, MMP-9 has an important role in cartilage destruction. This enzyme can break various...
plasma proteins and connective tissue such as proteoglycans, collagen type IV, collagen type V, collagen type VII, denaturation of collagen type I, laminine, fibronectin, and elastine (Soder et al, 2006). Recent studies has shown that MMP-9 play a role in the initiation of new blood vessel formations and remodeling of subcondral bone. Both are central in the process of OA (Gallaso et al, 2012).

**Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ)** is a transcription factor, a part of nuclear receptor superfamily involved in many cellular processes in the body. It is also expressed in chondrocyte cells. One study has found that the expression of in OA cartilage is lower compared to that of normal cartilage. It led to the hypothesis that the decreased PPAR-γ expression in OA cartilage is associated to the increased secretion of inflammation and catabolic factors (Afif et al, 2007). Other studies also found that adding PPAR-γ may decrease the syntheses of OA catabolic materials and inflammation factors as well as lowering cartilage degradation. **Peroxisome Proliferator-Activated Receptor gamma** can be activated by natural physiological agonists and synthetic agonists. Pioglitazone is one of several potent synthetic agonists of PPAR-γ (Kobayashi et al, 2005).

With the aforementioned basis, it was suggested that leptin and PPAR-γ have roles in pathogenesis of OA. However, their involvement in OA pathogenesis, specifically in chondrocytes cells, was yet to be studied. This study, therefore, aimed to identify the effect of leptin in inducing OA with a parameter of increased MMP-9 secretion as degradative enzyme by chondrocytes and whether it involve PPAR-γ.

2. Materials and Methods

This study used post-test only control group design with laboratory experimental design. The research was conducted for five months since April 2014 in several laboratories such as: Physiology Laboratory of Faculty of Medicine, Brawijaya University for chondrocyte line cells, ELISA MMP-9, and cDNA synthesis; Parasitology Laboratory of Faculty of Medicine, Brawijaya University RNA isolation; Laboratory of Life Science, Brawijaya University for nano drop; and Laboratory of Science and Technology, Maulana Malik Ibrahim University Malang for RT-PCR. The subject used was chondrocytes cell line, Normal human articular chondrocytes (NHAC-kn) from Lonza, which was divided into 7 (seven) treatment groups: 1) normal control group without any treatment; 2) a group exposed to IL-1β 10ng/ml only; 3) a group exposed to leptin10μg/ml; 4) a group exposed to IL-1β10ng/ml and leptin 100 ng/ml; 5) a group exposed to IL-1β10ng/ml and leptin 1 μg/ml; 6) a group exposed to IL-1β10ng/ml and leptin10μg/ml; and 7) a group exposed to IL-1β10ng/ml, leptin 10μg/ml and pioglitazone 10μM. Cells that reached 70-80% confluence were used for this research. The stages of the research were chondrocytes NHAC-kn culture, measurement of mRNA PPAR-γ expression using real time PCR via RNA isolation followed by reverse DNA transcription/synthesis, and the last was measurement of MMP-9 secretion using ELISA.

**Chondrocytes culture**

Thawing was conducted in sterile way in the laminar air flow according to the manual procedure of Lonza. Firstly, insert 1 ml of chondrocytes culture media (Bullet kit™, Lonza) into cryovial containing cryopreserved cell, then move them into 25cm² culture flasks containing media. Chondrocytes cells were incubated in 37°C and 5% CO₂, and the culture media were replaced with fresh media every 2 – 3 days until the cells were confluent. After chondrocytes cells reached 70-80% confluence, the cells were subcultured using chondrocytes ReagentPack™ subculture reagents. The media in the 25cm² culture flasks were disposed of before the flasks were rinsed with 5ml HEPES-BSS (Lonza) in room temperature. Afterward, HEPES-BSS was discarded and 3ml of trypsin/EDTA (Lonza) solution was added in room temperature. After stood for 2-6 minutes, the flasks were then observed using microscope while the cells were released slowly from the bottom using pipette. 6ml Trypsin Neutralizing Solution (Lonza) was added before the mixture were transferred into 15ml Falcon tubes and centrifuged in 1000rpm for 10 minutes. Supernatants were cleared and chondrocytes cells were obtained to be redifferentiated until the chondrocytes matured.

In this research, the redifferentiation process used alginate beads, as instructed by Lonza’s manual. The collected cells were soluted in 155mM NaCl and resuspended in 1.2% sodium alginate solution with 4x10⁵ sel/ml density. The cell/alginate mixture were added into CaCl₂ solution and stood for 10 minutes before the liquid was cleared and the beads were rinsed with 5x volume of 155mM NaCl 4 to 5 times, followed by twice cleansing using 1-2x volume of CDM™. The beads were placed into culture plate and added with 1 ml CDM™ per volume suspen of planted cell/alginate. The cells were incubated in 37°C and 5% CO₂ for 3-4 weeks. Media replacement was conducted every 3 or 4 days. After the cells reached 70 – 80% confluence, collagen type 2 levels were measured on the cultures’ supernatant using ELISA to determine whether cultured chondrocytes cells did not change into fibroblast-like cells.
Treatment and depolymerization of alginate beads

The cells were immediately treated after the increase of collagen type 2 was determined. IL-1β (Biolegend), leptin (R&D), and pioglitazone (Sigma) were added when the cells were still within the beads and the administration was conducted in sterile condition in the laminar air flow. IL-1β 10ng/ml was firstly added and 24 hours incubation was conducted. The administration of leptin and pioglitazone were given afterward, followed by another 24-hour incubation. After treatment and measurement, supernatants were collected for measurement of MMP-secretion using ELISA. The cells inside the beads were depolymerized by adding 3x volume of 55mM Na Citrate for 20-30 minutes in room temperature. The cells were rinsed two times using 155mM NaCl. The cell pellets were soaked in TRI Reagent solution and stored in -40ºC before RNA isolation.

Measurement of PPAR-γ expression

a) RNA isolation

Cell cultures were homogenized in 1mL TRI Reagent solution for every 5 x 10⁶ cells, or every 10 cm² culture area, before incubation for 5 minutes in room temperature. 100μl of of chloroform was added afterward, followed another incubation for 5 minutes in room temperature. The solution was centrifuged at 12,000g for 10 minutes at 4 °C, and then the aqueous phase was transferred to a new tube. Added 250μl of isopropanol and incubated at room temperature for 5-10 minutes. Then centrifuged at 12,000g for 8 minutes at 4-25 °C, and the supernatant was discarded and 500μl of 75% ethanol added. The solution was then centrifuged on 7,500g for 5 minutes on 4-25°C, followed by removal of ethanol and drying of RNA pellet. Lastly, RNA was dissolve into Nuclease-free Water and stored in -40°C before RNA isolation.

b) cDNA synthesis

CDNA in this research was synthesized using GoScript™ Reverse transcription System kit (Promega, Cat. A5000). 0.5μg of RNA and 0.25μg of Oligo (dT)₁₅ primer were mixed with Nuclease-Free Water until it reached 2.5μl of volume in the RNA tubes. The tubes were stored in PCR machine in 70°C for 5 minutes before quickly moved into chill ice for 5 minutes. Each tube was centrifuged for 10 seconds. Reverse transcription reaction mix was prepared for all reactions. It consisted of GoScript™ 5X 30μl of reaction buffer, 7.5μl of GoScript™ reverse transcriptase, 45μl of MgCl₂, 7.5μl of PCR nucleotide mix, and 22.5μl of nuclease free water. The reaction mix was added into sample tubes, followed by pipetting and spinning down. The tubes then stored in PCR machine in 25°C for 5 minutes for annealing, 42°C for 60 minutes, and 70°C for 15 minutes. Lastly, the samples were stored in chill ice in -20°C.

c) Real Time Polymerase Chain Reaction

Reagents used in real time PCR (LightCycler SYBR Green, Roche) were prepared and mixed for all reactions. Vials containing enzyme and vials containing reaction mix were combined, followed by pipetting and spinning down, and then labeled as master mix. The master mix were added with PPAR-γ primer and GAPDH primer both forward and reverse before water PCR grade was administered. Primer used in this research (Wang et al, 2012).

PPAR-γ:
F 5 – TGA CCA GGG AGT TCC TCA AAA – 3
R 5 – AGCAAAACTCAAACCTTAGGCTCCAT– 3;

With internal control using GAPDH (Nebbaki et al, 2012)

GAPDH :
F 5 – CAG AAC ATC ATC CCT GCC TCT – 3
R 5 – GCT TGA CAA AGT GGT CGT TGA – 3

7,5μl of master mix was inserted into cold LightCycler Capillary, added with 2,5μl of DNA template sample and spun down. The capillaries were stored in real time PCR machine (LightCycler 1. 5 Roche) for a cycle of pre-incubation in 95°C for 10 minutes; for amplification consisting 45 cycles of 10-second denaturation in 95°C, 20-second annealing in 56°C, and 25-second extension in 72°C; for a cycle of melting curve analysis consisting 0-second denaturation in 95°C, 15-second-annealing in 65°C, 0-second melting in 95°C with 0.1°C/second slope; and 30-second cooling in 40°C. The results were analyzed qualitatively and quantitatively by comparing it with standard curve.

Measurement of MMP-9

MMP-9 secretion was measured using MMP-9 (Human) ELISA kit (R&D System, no kat DMP900). The first step was standard formation. 6 endorf tubes were filled with 500μl of Calibrator Diluent RD5-10 each. The first tube was added 500μl of the highest standard (20 /ml). After mixing and vortex, 500μl of solution from the first tube was taken and put into the second tube. This step was repeated until the last tube.

Next, 100μl of Assay Diluent RD1-34 was inserted into each well. After addition of 100μl of standard and sample on the established wells, they were sealed with adhesive strip and incubated for 2 hours on a shaker. Once incubation was ended, each well was aspirated and washed using wash buffer for three times. 200μl of MMP-9 Conjugate was added afterward and incubation was conducted place for an hour. After washing, 200μl of Substrate Solution was added and the last incubation in a dark place for 30 minutes was done. Last, 50μl of stop solution was
added. Absorbance was read using ELISA reader on 450nm.

Data Analysis

Figure 1. The amount of MMP-9 secretion in all treatment groups. The measurement used ELISA. It is shown that induction of IL-1β increased MMP-9 secretion significantly, compared to normal group (p < 0.05). Normal is untreated chondrocytes cells; IL-1B is chondrocytes cells exposed to 10ng/ml of IL-1β for 24 hours. Error bar in the histogram shows average ± standard error from the mean. Difference is significant if p < 0.05.

In this research, two data groups were obtained i.e. data of PPAR-γ expression and MMP-9 level. Data were tested using one way Anova to determine the differences in the group. To know which group is different, post hoc Tukey was used for further test. Afterwards, correlation test were taken to determine the correlation between leptin and MMP-9 level or PPAR-γ expression. Degree of confidence was 95% (α = 0.05), it was significant if p < 0.05.

3. Result and Discussion

Effect of Leptin Addition on MMP-9 Secretion by IL-1β-induced Chondrocytes

Leptin has been included as cytokine-like hormone because its structure is similar to that of cytokine and its receptor is similar to class I cytokine family’s receptors (Otero et al, 2005). This hormone’s proinflammation trait has been proved through several researches. Data has shown that leptin may not only increase secretion of proinflammation cytokines in the experimental animals, but also involved in the regulation of immune response both humoral and cellular in arthritic model experimental animals (Busso et al, 2002).

The results of this research suggested that the addition of leptin to IL-1β-induced chondrocytes were able to increase MMP-9 secretion, and this increase was equivalent to the added dose of leptin. These results were in accordance with other studies in terms of leptin’s capability in increasing MMP-9 secretion in various cells. The addition of leptin into spinal cord of rodent in vitro was able to increase the expressions of NADPH oxidase-2 (NOX2) and MMP-9 (SchroeTer et al, 2011). The addition of leptin was also increase MMP-9 and E-caderin expressions in in vitro placenta cells model (Jo et al, 2004). Other research results suggested that leption addition via intraarticular injection in rodent might increase MMP-2 and MMP-9 expressions in cartilage cells significantly (Bao et al, 2010).

Matrix metalloproteinase-9 has an important role in the initiation of blood vessel formation and remodeling of subcondral bone. To create a new blood vessel branch, the membrane basalis must be deformed first. The main structure in the formation of membrane basalis is collagen type IV. Collagen type VI is degraded by MMP-2 and, mainly, MMP-9. This degradation is a central process in the beginning of capillaries formation (Xiang and Sang, 1998).

From the previous result, it has been shown that IL-1β induction may change the metabolic trait of chondrocytes into catabolic. Leptin addition has also proven to increase this effect. This suggested that leptin might act as a proinflammation factor in the cartilages. Obesity is one of OA risk factors, where the increase in leptin level is proportional to the weight gained by obese people. The results in this research support the theory that not only obesity that trigger OA, in terms of
mechanical weight, but also the involvement of leptin in the mechanism of cartilage destruction. In this research, IL-1β was used with the dose of 10ng/ml. This referred to another research wherein 10ng/ml of IL-1β was used to induce MMP-13 production in normal chondrocytes (Mengshol et al, 2000). A different research suggested that the exposure of 100pg/ml of IL-1β was able to induce MMP-1 and MMP-13 significantly (Zayed et al, 2008). These findings suggested that IL-1β exposure with different doses results in the secretion of MMP of different level as well. Therefore, a further research is needed, exclusively using various doses of IL-1β and leptin to determine which level of IL-1β and leptin that may highly induce MMP-9 secretion.

The Effect of Leptin to the Expression of PPAR-γ

The effect of PPAR-γ on chondrocytes is condroprotective as it suppresses the progress of OA. The decreased PPAR-γ expression in cartilage will increase inflammation and catabolic gen expressions, leading to articular inflammation and cartilage degradation (Fahmi et al, 2011). In this research, the results suggested that the induction of IL-1β on chondrocytes might decrease PPAR-γ expression significantly.

This result is compliant with the results of several preceding researches. A research conducted by Mracek et al suggested that the addition of IL-1β may inhibit adipose cells differentiation, followed by decreasing mRNA PPAR-γ expression (Mrácek et al, 2004). This PPAR-γ suppression by IL-1β occurred via downstream pathway from IL-1β signalling i.e. TAK1/TAB1/NF-κB-inducing kinase (NIK) cascade (Suzawa et al, 2003).

Further results in this research suggested that the addition of leptin may decrease PPAR-γ expression significantly compared to that of normal chondrocytes. Adding leptin to IL-1β-induced chondrocytes showed an interesting result. On low and medium dose of leptin, PPAR-γ expressions did not differ with the expression of PPAR-γ on group which were induced only by IL-1β, but on high dose the PPAR-γ expression decreased significantly.

Several results on the effect of leptin on PPAR-γ expression were contradicting. Early researches have shown that leptin addition may increase PPAR-γ expression in adipose tissue. Qian et al’s research (1998) in vivo on rodents supported this. Leptin was administered via intracerebroventricular (ICV) and the PPAR-γ expression was measured after 5 days. The results suggested that leptin has increased PPAR-γ expression about 70-80% (Qian et al, 1998). A more recent research, however, showed the opposite. A research by Zhou et al (2009) suggested that leptin addition may decrease PPAR-γ expression in rodents stellata cells via activation of ERK1/2 pathway. Other in vivo research on macrophage cells in human showed that leptin addition could decrease PPAR-γ expression in macrophage and foam cells (Li et al, 2006).

A research using pig suggested that high dose administration of leptin may decrease PPAR-γ expression in adipocytes (Ajuwon et al, 2003). A research on chondrocytes culture showed that leptin-activated MAPK/ERK signals were involved in the
inhibition of PPAR-γ activities and negatively affected the PPAR-γ (Sebastian et al, 2011). A research on rodent chondrocytes showed that addition of leptin gave PPAR-γ suppression effect on the condrogenic differentiation and terminal differentiation in chondrocytes. Leptin were able to decrease PPAR-γ expression via phosphorylation mechanism in serin 112/82 mediated by MAPK/ERK. Leptin addition decreased only a little PPAR-γ on mRNA level, so it was suggested that leptin may also suppress the activity of PPAR-γ posttranslational modification (Wang et al, 2012).

In the current research, the administration of leptin was able to decrease PPAR-γ significantly. This decrease was also positively associated to the secretion level of MMP-9. From these results, it was suggested that leptin may increase MMP-9 secretion and thus followed by the decrease of PPAR-γ expression.

The Effect of Pioglitazone on MMP-9 Secretion by IL-1β- and leptin-induced Chondrocytes

In the current research, the PPAR-γ agonists were involved to determine the role of PPAR-γ in chondrocytes metabolism, specifically in terms of MMP-9 production. Another research suggested that the activation of PPAR-γ by its agonists may decrease the synthesis of catabolic components and inflammation factors as well as lessening cartilage degradation in OA model experimental animals (Fahmi et al, 2011). However, research conducted to identify the effect of PPAR-γ on MMP-9 production has yet to be done.

To regulate gene transcriptions, PPAR-γ may directly involve in DNA binding or interacting with other transcription factors. For instance, PPAR-γ may directly interacting with proinflammation transcription factors such as activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and NF-kB. A proinflammation transcription factor, NF-kB, has a central role in immune response and inflammation, where NF-kB becomes the main target of PPAR-γ in suppressing inflammation (Duan et al, 2008).

In this research, leptin has been proved able to reduce PPAR-γ expression significantly. This decrease was associated to the increased MMP-9 secretion. Administration of pioglitazone as PPAR-γ agonists were able to decrease MMP-9 secretion significantly in IL-1β- and leptin-induced chondrocytes. These results suggested that the increase of MMP-9 secretion in IL-1β- and leptin-induced chondrocytes was truly mediated by PPAR-γ.

The addition of pioglitazone may also decrease MMP-9 secretion significantly. Several researches have also proven that pioglitazone significantly decreased the main mediator in OA, that were, MMP-1, ADAMTS-5, and iNOS (Boileau et al, 2007). This explained the potential use of pioglitazone as a new therapy for OA. A further research, nevertheless, using various doses is required to determine the effect on OA as a new alternative for therapy, since in this research only one dose of pioglitazone was used.

4. Conclusion

Leptin was able to increase MMP-9 secretion through chondrocytes induced by IL-1β via PPAR-γ suppression. The drawback in this research was the use of chondrocytes cell line cultures as research subject. Cell lines were favorable because they were easily cultured and the chondrocytes cells were controllable, in terms that they were only affected by the treatments received during research. On the other hand, cell line were immortal cells that genetically have differences with chondrocytes cells in vivo, and therefore the observed results might be different from those that use primary culture cells as research subject (Bedetti and Cantafora, 1990).

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