ejpmr, 2016,3(1), 40-45

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-Γ (PPAR-Γ) AGONIST INDUCE SENSITIZATION B1 ADRENERGIC RECEPTOR VIA REGULATION EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 (ERK 1/2) AND G PROTEIN-COUPLED RECEPTOR KINASE-2 (GRK-2) IN CULTURE CARDIOMYOCYTE

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Article Received on 05/11/2015 **Article Revised on 26/11/2015** **Article Accepted on 16/12/2015**

ABSTRACT

The present study evaluated effect of pioglitazone (PPAR γ agonist) to sensitize β1-adrenergic receptor in culture cardiomyocyte. Up-regulated GRK2 and down-regulated of β1-adrenergic receptor in the heart underlies the diminished contractile responsiveness of the heart to positive inotropes. The result of study showed that potention of pioglitazone to increase or sensitize β1-adrenergic receptor via increase ERK1/2 and decrease GRK-2. The potention pioglitazone to increase concentration of β1-adrenergic receptor not only based on dose-dependent of pioglitazone but also influenced by concentration of norepinephrine.

KEYWORDS: Pioglitazon – PPAR γ Agonist – β1 adrenergic – GRK2 – ERK1/2.

BACKGROUND

Despite significant advances in pharmacological and clinical treatment, heart failure (HF) remains the number one killer disease in the world. HF is a chronic and progressive clinical syndrome mainly characterized by reduction in left ventricular ejection fraction and adverse remodeling of the myocardium. One of its hallmark molecular abnormalities is elevation of cardiac G protein-coupled receptor (GPCR) kinase (GRK)-2, a member of the GRK family of serine/threonine protein kinases which phosphorylate and desensitize GPCRs (β1-adrenergic receptor). Up-regulated GRK2 and down-regulated of β1-adrenergic receptor in the heart underlies the diminished contractile responsiveness of the heart to positive inotropes.[1]

G protein-coupled receptor kinases (GRKs) were initially identified as serine/threonine kinases that participate together with arrestins in the regulation of multiple GPCR. The GRKs constitute a group of protein kinases (seven members in mammals) that specifically recognize and phosphorylate agonist-activated GPCRs.[2-10] Arrestins then bind to the phosphorylated receptor, leading to uncoupling from heterotrimeric G proteins and receptor desensitization. As a result of β-arrestin binding, phosphorylated receptors are also targeted for clathrin-mediated endocytosis, a process that classically serves to re-sensitize and recycle receptors back to the plasma membrane.[11-17]

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily, are ligand-activated transcription factors that play an important role in lipid metabolism.[18-20] One of these PPARs, PPARγ, has been implicated in the transcriptional regulation of several genes involved in lipid metabolism and appears to promote the differentiation of cells toward a more adipocyte-like phenotype.[21-24] Both synthetic and natural ligands for PPARγ have been described. Among the synthetic ligands, thiazolidinediones, a group of compounds that includes pioglitazone, increase insulin sensitivity.[25] Naturally-occurring PPARγ ligands include fatty acids, eicosanoid derivatives and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2).[26] Recent work has established that 9-hydroxy-(S)-10,12-octadecadienoic acid [9(S)-HODE] and 13(S)-HODE, known components of oxidized LDL, are PPARγ activators, with concomitant evidence invoking PPARγ signaling in monocytes and macrophages.[27] Clinical observations suggest that obese patients have elevated adipose tissue PPARγ levels compared with those in lean controls. Once activated, PPARγ binds to the PPAR response elements (PPRE) in the promotor region of target genes.[27]

Bolden et al. demonstrated that troglitazone (PPAR γ agonist) induce ERK phosphorylation in human prostate cancer cells via a PPARγ-independent signaling pathway.[28] Inhibition of ERK activity in HEK293 cells potentiates GRK2 activity, whereas, conversely, ERK activation inhibits GRK2 activity. The discovery that

ERK phosphorylates and inactivates GRK2 suggests that ERK participates in a feedback regulatory loop.[29]

METHODS

Materials

Culture medium

One packet of DMEM powder(GIBCO, 12800-017) is dissolved in 1000 ml of distilled water, 2.2 g sodium bicarbonate and 25 mM Hydroxyethyl piperazine ethanesulfonic acid (HEPES) (Sigma, H4034) are added and adjust the pH value to 7.3. This solution is sterilized by filter-sterilization. The following sterile solutions are added to complete the DMEM: 10% (v/v) heat-inactivated fetal bovine serum (Hang Zhou Sijiqing, 050416), Antibiotics stock mixture (Hyclone, SV30010). Enzyme solution: 0.08% trypsin solution is freshly prepared to ensure the enzyme activity. Dissolve trypsin (Sigma, T8003) in Ca2+ and Mg2+-free PBS buffer either at room temperature for 4 hour with agitation or at 4^{o}C overnight. Sterilize the solution by filtration through a 0.22 µm filter.

PBS solution

one packet of Ca2+ and Mg2+- free PBS powder (Boster, AR0030) is dissolved in 2 L dH2O. The pH value is adjusted to 7.3. The solution is sterilized either by autoclaving or filter sterilization. Deoxyribonuclease I (sigma, 31135) is dissolved in sterile PBS buffer to make a stock solution of 5 mg/ml, dilute the solution to 50 µg /ml with trypsin solution before use. 5-Bromo-2-deoxyuridine (BrdU) (Aldrich, 858811) is dissolved in sterile dH2O to a stock solution of 100 mmol/l, final concentration is 0.1 mmol/ l. Cell culture plates (diameter 35 mm) and flasks (Greiner bio-one, 657 160, 690 170 respectively).

Rat Pups and Evacuation of Heart

Three-day-old rat pups Spraque-dawley (ten rat pups) were obtained from Laboratory Research Unit Gadjah Mada University, Yogyakarta, Indonesia, with approval of the Ethics Committee of Laboratory Research Unit Gadjah Mada University, Yogyakarta, Indonesia.

The animals were decontaminated with 75% ethanol and transferred to a Luminar air flow. Dissection (performed on ice), surgically remove the beating heart from animals immediately, and keep it in cold Ca2+ and Mg2+-free PBS buffer. Ventricles were excised and transferred to fresh ice-cold PBS buffer and were minced with fine scissors into 1–3 mm^3 pieces after washing blood away from the heart lumen.

Culture of Cardiomyocyte[30]

Preconditioning (20 min): A preconditioning step was introduced in the present protocol prior to trypsinization. The minced tissue was transferred to a 40 ml conical flask containing trypsin solution (0.08%, 0.5 ml per rat) and a small magnetic bead. The flask was then settled on ice for 20 min, and shaken every 3 min for better mixing.

Trypsinization (10 min): Following preconditioning, the tissue was digested in the conical flask at 37^{o}C for 10 min, which was subjected to constant stirring (150–200 rpm).

Centrifugation (5 min): After trypsinization, cells were dispersed from the tissue by gently pipeting. The cell suspension was settled on ice for several seconds. The supernatant was carefully transferred to a 15 ml centrifuge tube. Trypsin activity was inhibited by adding a mixture of trypsin inhibitor and cold culture medium supplemented with 10% FBS (1:1, v/v). Cell pellet was formed by spinning at 1000 rpm for 5 min, and was resuspended in 2 ml warm culture medium.

Repeating trypsinization: The remaining tissue in the conical flask was continuously digested by adding 5–10 ml fresh pre-warmed trypsin solution containing DNase I (0.05 mg /ml). Depending on the amount of undigested tissue, trypsinization and centrifugation steps were repeated for 2 to 3 times (25–35 min).

Cell harvest (10 min): Cell suspension from all dissociated steps was pooled in one centrifuge tube and settled for 5 min. The suspension was gently transferred to a new centrifuge tube excluding the precipitates on the bottom. Cells were harvested by centrifugation for 5 min at 1000 rpm. Finally the cells were plated in a 40 ml tissue culture flask and incubated at 37^{o}C in a humidified atmosphere (5% CO2, 95% air).

Purification (1.5 hours): Myocardiocytes enriched culture is obtained through the following two steps. Since non-myocardiocytes attach to the substrata more readily than myocardiocytes, firstly, cells harvested were incubated for 1.5 h to allow the attachment of non-myocardiocytes. The majority of myocardiocytes remained in culture medium. The suspended cells were collected and plated at a density of 2 x 10^5/ml into a new tissue culture flask. BrdU (0.1 mmol/l) was then added to the culture medium for 48 h to prevent proliferation of nonmyocardiocytes that might be present in the culture.

Cultivation

Generally, cells isolated from 2 to 3 hearts can be seeded in one 40 ml culture flask. The cells should not be disturbed during the initial 24 h. The culture medium was replaced with fresh media without BrdU for every 48 h.

The yield and viability of the culture was monitored by dye exclusion using trypan blue (0.4%). Mix 1 drop of trypan blue (0.4%) with 9 drops of cell suspension and allow 1–2 min for absorption. Cells excluding the staining are considered viable and the percentage of non-blue cells is used as an index of viability. Count both the total number of cells and the number of stained (dark) cells by a hemocytometer for measuring the yield and viability as follows.

Yield = (total number of cells in four grids/4) x 10^4 x (cell suspension volume).
Viability (%) = (Total cells counted - stained cells)/total cells counted x 100.

Norepinephrin (two gradients concentration 10 µmol/L or 100 µmol/L) was added to cardiomyocyte culture for 24 hour. Norephinephrin was added to desensitize β1 adrenergic receptor, as condition of heart failure.

Cardiomyocyte cells were randomized into nine groups. Group 1:5 plates (every plate contain: 2 x 10^5/mL cells) were not added norepinephrin and pioglitazone. Group 2: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 10 µmol/L. Group 3: 5 plates (every plate contain: 2 x 10^5 /mL cells) were added norephinephrin 100 µmol/L. Group 4: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 1 µmol/L. Group 5: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 10 µmol/L. Group 6: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 100 µmol/L. Group 7: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 1 µmol/L. Group 8: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 10 µmol/L. Group 9: 5 plates (every plate contain: 2 x 10^5/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 100 µmol/L.

β1-Adrenergic Receptor Analysis
Culture media was removed and Cells were washed with ice cold-PBS (pH 7,2-7,4). Cells was removed and incubated in -20^{o}C for a night. Cells was centrifuge for 5 minutes, 5000 x g, 2-8^{o}C. Supernatan was collected for ELISA assay.

Solid phase sandwich ELISA (Rat β1 adrenergic receptor ELISA kit, elabscience) were used to analysis concentration of β1 adrenergic receptor. . Add samples and standards and incubate the plate at 37^{o}C for 90 minutes, do not wash. Add biotinylated antibodies and incubate the plate at 37^{o}C for 60 minutes, wash plate 3 times with PBS 0,01 M. Add HRP working solution and incubate the plate at 37^{o}C for 30 minutes. Wash plate 5 times with PBS 0,01 M. Add HRP colour developing agent and incubate the plate at 37^{o}C in dark for 20 minutes. Add stop solution and read the OD absorbance at 450nm in a microplate reader. The standard curve was plotted as the OD 450 of each standard solution vs the concentration of standard solution. The rat β1 adrenergic receptor concentration of the samples was interpolated from the standard curve.

GRK-2 Analysis
Culture media was removed and Cells were washed with ice cold-PBS (pH 7,2-7,4). Cells was removed and incubated in -20^{o}C for a night. Cells was centrifuge for 5 minutes, 5000 x g, 2-8^{o}C . Supernatan was collected for ELISA assay.

Solid phase sandwich ELISA (Rat GRK-2 ELISA kit, elabscience) were used to analysis concentration of GRK-2 . Add samples and standards and incubate the plate at 37^{o}C for 90 minutes, do not wash. Add biotinylated antibodies and incubate the plate at 37^{o}C for 60 minutes, wash plate 3 times with PBS 0,01 M. Add HRP working solution and incubate the plate at 37^{o}C for 30 minutes. Wash plate 5 times with PBS 0,01 M. Add HRP colour developing agent and incubate the plate at 37^{o}C in dark for 20 minutes. Add stop solution and read the OD absorbance at 450 nm in a microplate reader. The standard curve was plotted as the OD 450 of each standard solution vs the concentration of standard solution. The rat GRK-2 concentration of the samples was interpolated from the standard curve.

ERK 1/2 Analysis
Culture media was removed and Cells were washed with ice cold-PBS (pH 7,2-7,4). Cells was removed and incubated in -20^{o}C for a night. Cells was centrifuge for 5 minutes, 5000 x g, 2-8^{o}C . Supernatan was collected for ELISA assay.

Solid phase sandwich ELISA (Rat ERK 1/2 ELISA kit, elabscience) were used to analysis concentration of ERK1/2. Add samples and standards and incubate the plate at 37^{o}C for 90 minutes, do not wash. Add biotinylated antibodies and incubate the plate at 37^{o}C for 60 minutes, wash plate 3 times with PBS 0,01 M. Add HRP working solution and incubate the plate at 37^{o}C for 30 minutes. Wash plate 5 times with PBS 0,01 M. Add HRP colour developing agent and incubate the plate at 37^{o}C in dark for 20 minutes. Add stop solution and read the OD absorbance at 450nm in a microplate reader. The standard curve was plotted as the OD 450 of each standard solution vs the concentration of standard solution. The rat ERK1/2 concentration of the samples was interpolated from the standard curve.

Statistics
Bivariate analysis for concentration of ELISA was analyzed by T-test. Multivariate analysis for concentration of ELISA was analyzed by one way ANOVA, followed pos hoc analysis with bonferroni test. A value of $p < 0,05$ was considered stastistically significant.

RESULTS

β1-Adrenergic Receptor Concentration
Pioglitazone increased concentration of β1-adrenergic receptor in group 8 and 9, compared to group 3 (only norepinephrin 100 µmol/L), $p< 0,05$. But, Pioglitazone did not increase concentration of β1-adrenergic receptor in group 4,5 and 6, compared to group 2 (only norepinephrin 10 µmol/L), $p<0,05$.

Table 1. Concentration of β1-Adrenergic Receptor.

Group	Concentration (pg/mL) ± SD
1	1219,66 ± 78,98
2	921,46 ± 56,34
3	700,92 ± 65,87
4	497,72 ± 45,67
5	500,94 ± 52,34
6	894,22 ± 88,87
7	298,78 ± 32,12
8	906,55 ± 65,45
9	2442,28 ± 76,99

Group 1:5 plates (every plate contain: 2 x 105/mL cells) were not added norepinephrin and pioglitazone. Group 2: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L. Group 3: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L. Group 4: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 1 µmol/L. Group 5: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 10 µmol/L. Group 6: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 100 µmol/L. Group 7: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 1 µmol/L. Group 8: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 10 µmol/L. Group 9: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 100 µmol/L.

GRK-2 Concentration

Pioglitazone decreased concentration of GRK-2 in group 5 and 6, compared to group 2, p<0,05. Concentration of GRK-2 decreased after added pioglitazone in group 8 and 9 compared to group 3, p<0,05.

Table 2. Concentration of GRK-2.

Group	Concentration (pg/mL) ± SD
1	689,16 ± 48,98
2	642,56 ± 86,34
3	799,52 ± 65,87
4	897,62 ± 65,67
5	600,54 ± 62,34
6	498,22 ± 78,87
7	831,18 ± 62,12
8	608,55 ± 35,45
9	442,38 ± 46,99

Group 1:5 plates (every plate contain: 2 x 105/mL cells) were not added norepinephrin and pioglitazone. Group 2: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L. Group 3: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L. Group 4: 5 plates (every plate contain: 2 x 105 /mL cells) were added norephinephrin 10 µmol/L and pioglitazone 1 µmol/L. Group 5: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 10 µmol/L. Group 6: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 100 µmol/L. Group 7: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 1 µmol/L. Group 8: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 10 µmol/L. Group 9: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 100 µmol/L.

ERK 1/2 Concentration

Pioglitazone increased concentration of ERK 1/2 in group 6 compared to group 2, p<0,05. Concentration of ERK1/2 increased after added pioglitazone in group 9 compared to group 3, p< 0,05.

Table 3. Concentration of ERK1/2.

Group	Concentration (pg/mL) ± SD
1	759,46 ± 68,68
2	632,46 ± 76,34
3	519,52 ± 65,87
4	397,62 ± 35,67
5	500,54 ± 42,34
6	698,22 ± 78,87
7	231,18 ± 62,12
8	408,55 ± 35,45
9	552,28 ± 46,99

Group 1:5 plates (every plate contain: 2 x 105/mL cells) were not added norepinephrin and pioglitazone. Group 2: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L. Group 3: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L. Group 4: 5 plates (every plate contain: 2 x 105 /mL cells) were added norephinephrin 10 µmol/L and pioglitazone 1 µmol/L. Group 5: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 10 µmol/L. Group 6: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 10 µmol/L and pioglitazone 100 µmol/L. Group 7: 5 plates (every plate contain: 2 x 105 /mL cells) were added norephinephrin 100 µmol/L and pioglitazone 1 µmol/L. Group 8: 5 plates (every plate contain: 2 x 105/mL cells) were added norephinephrin 100 µmol/L and pioglitazone 10 µmol/L. Group 9: 5 plates (every plate contain: 2 x 105 /mL cells) were added norephinephrin 100 µmol/L and pioglitazone 100 µmol/L.

DISCUSSION

Norepinephrine was confirmed to decrease concentration of β1-adrenergic receptor, desensitize β1-adrenergic receptor. Norepinephrine also was confirmed to increase concentration of GRK-2 and decrease ERK1/2. It was confirmed that norepinephrine has a potention to

desensitize β1-adrenergic receptor via increase concentration of GRK-2 and decrease ERK1/2. Furthermore, Choi et al., shows the effect of norepinephrine to desensitize or decrease activity of β1-adrenergic receptor and increase concentration of GRK-2.[29] Yaniv et al., shows the effect of norepinephrine to increase concentration of ERK1/2. [31]

Pioglitazone (PPAR γ agonist) was confirmed had a potention to decrease and increase concentration of β1-adrenergic receptor. Pioglitazon decreased concentration of β1-adrenergic receptor in the group which added norepinephrine 10 µmol/L, compared to group which only added norephineprine 10 µmol/L without pioglitazone. But, in the group which added norepinephrine 100 µmol/L, pioglitazone 10 and 100 µmol/L increased concentration of β1-adrenergic receptor compared to group which only added norepinephrine 100 µmol/L. The potention pioglitazone to increase concentration of β1-adrenergic receptor not only based on dose-dependent of pioglitazone but also influenced by concentration of norepinephrine.

Pioglitazone was confirmed to increase concentration of ERK1/2. The potention pioglitazone to increase concentration of ERK1/2 based on dose-dependent effect phenomenon, but not influenced by concentration of norepinephrine. Bolden et al. demonstrated that troglitazone (PPAR γ agonist) induce ERK phosphorylation in human prostate cancer cells via a PPARγ-independent signaling pathway.[28]

Inhibition of ERK activity in HEK293 cells potentiates GRK2 activity, whereas, conversely, ERK activation inhibits GRK2 activity. The discovery that ERK phosphorylates and inactivates GRK2 suggests that ERK participates in a feedback regulatory loop.[29] Pioglitazone was confirmed to decrease concentration of GRK-2. The potention pioglitazone to decrease concentration of GRK-2 based on dose-dependent effect phenomenon, but not influenced by concentration of norepinephrine.

It is postulated that the potention of pioglitazone to increase or sensitize β1-adrenergic receptor via increase ERK1/2 and decrease GRK-2. The potention pioglitazone to increase concentration of β1-adrenergic receptor not only based on dose-dependent of pioglitazone but also influenced by concentration of norepinephrine. There is a potention another pathway or another mechanism that influenced the effect pioglitazone to increase or sensitize β1-adrenergic receptor.

ACKNOWLEDGMENTS

This study was supported by research foundation program (Risbin iptekdok 2015) Ministry of Health Indonesia. We thank to reviewer team from Risbin iptekdok 2015.

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