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Neng F. Kurniati  
Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia

Teresa Angelia  
Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia

Aluicia A. Artarini  
Department of Pharmaceutical, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia

Maritsa Nurfatwa  
Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia, maritsa.nurfatwa@gmail.com

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Myocardial Infarction Elevates Inflammation and Contributes to the Formation of Atheroma Plaques in the Aorta of Hypercholesterolaemic Rats

Neng F Kurniati1, Teresa Angelia1, Aluicia A Artarini2, Maritsa Nurfatwa1*

1. Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia
2. Department of Pharmaceutical, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia

*E-mail: maritsa.nurfatwa@gmail.com

Abstract

Background: Myocardial infarction (MI) is the clinical manifestation of coronary heart disease that can be caused by atherosclerotic plaque rupture. However, the role of MI in influencing endothelial cells is still not clearly known, especially in atheroma plaque development. The aim of this study was to determine the effect of MI in the inflammatory processes occurring in the hypercholesterolaemic rat aorta and heart by measuring myeloperoxidase (MPO) levels.

Methods: Wistar rats were categorised into normal, normal–MI, hypercholesterolaemic and hypercholesterolaemic–MI groups. Hypercholesterolaemia was induced in rats by feeding them with a high-cholesterol diet, followed by oral administrations of cholesterol, cholic acid and propylthiouracil. The MI rat model was created by injecting isoproterenol (intraperitoneal) 1 day before the animals were sacrificed. The success of the induction was confirmed based on a significant increase in total cholesterol values compared to those in the normal group. The inflammatory condition was determined by measuring the MPO levels using the dot blot method. Results: MPO expression was increased significantly in the hypercholesterolaemic rats compared to that in the normal group. The highest aorta MPO expression was observed in the hypercholesterolaemic–MI group. Both MI rats and hypercholesterolaemic rats showed a similar increase in MPO expression in the heart (71.7% and 75.5%, respectively). However, the hypercholesterolaemic–MI rats showed the highest MPO expression (119.59%). Conclusions: MI accelerates inflammation in the aorta of hypercholesterolaemic rats.

Keywords: atherosclerosis, hypercholesterolaemic, myeloperoxidase, myocardial infarction

Introduction

Myocardial infarction (MI) can be caused due to genetic factors, diseases and environmental reasons. Hypertension and diabetes mellitus can increase the risk of coronary heart disease. Smoking can also increase the prevalence of MI. Other factors include lack of exercise, high consumption of saturated fats and lack of consumption of certain vitamins. High levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol have been reported to be consistently associated with the risk of coronary heart disease.1

Oxidised LDL mediates the transformation of macrophages to cholesterol-rich foam cells. If left untreated, cloggings occurring in small and medium blood vessels in the inferior extremity may result in loss of sensation and gangrene (tissue death) in the legs. Atheroma plaque formation in the blood vessels can cause heart attacks and strokes.2 The diagnosis of hypercholesterolaemia can be established through several blood parameters such as total cholesterol, triglycerides, HDL and LDL. High levels of total cholesterol, triglycerides and LDL, as well as low levels of HDL, are among the factors that can induce the formation of atheroma plaques.

Inflammatory conditions play a major role in stages of atheroma plaque formation. Inflammatory mediators such as oxidants and free radicals are produced by various cells in the body as a mechanism of resistance to disease-causing agents. Myeloperoxidase (MPO) is an enzyme released by activated and degranulated polymorphonuclear neutrophils and monocytes. MPO is released by leukocytes under inflammatory conditions and catalyses the production of some reactive oxidant species such as hypochlorite acid (HOCI), which contribute towards protection against microbiorganisms.3 MPO along with other enzymes such as lipoxygenase can initiate lipid oxidation in the subendothelial area of the blood vessel wall. MPO also participates in endothelial dysfunction, because MPO uses nitrogen oxide as a substrate. The most important characteristic of MPO and its end product, HOCI, is the ability to activate matrix metalloproteinases.
(MMPs) and deactivate MMP inhibitors, which further weaken the fibrous cap and cause instability of the atheroma plaque. Therefore, MPO may be considered as one of the markers in the formation and development of atheroma plaques. Patients with coronary heart disease have been reported to show an increase in MPO levels in the blood. Therefore, MPO can be used to determine disease progression of atheroma plaques and MI.

The role of MI in the development and rupture of atheroma plaques is still not clear. Therefore, in this study, the role of MI in the formation of atheroma plaques was investigated, for which hypercholesterolaemia was induced by orally feeding the test animals with food rich in cholesterol, propylthiouracil (PTU), pure cholesterol and cholic acid. Furthermore, MI was induced in the animals through the administration of isoproterenol intraperitoneally the day before sacrifice. MPO levels in the aortic blood vessels and the heart of the animals were determined using the dot blot method. The aim of this study was to determine the effect of hypercholesterolaemia in the inflammatory process occurring in the aorta and the heart under the condition of MI through the measurement of MPO expression.

Methods

Materials. The following materials were used in this study: high-cholesterol Chow 10 kg (20 pieces of chicken liver, 1 kg goat fat, 75 quail eggs, 20 duck eggs, 3 kg wheat flour and 2 kg corn flour), normal chow, cholic acid, pure cholesterol, PTU, vegetable oil, aquadest, NaCl 0.9% solution, CMC-sodium, total cholesterol kit (supplier PT. Rajawali Nusindo, Jakarta, Indonesia), HDL kit (supplier PT. Rajawali Nusindo, Jakarta, Indonesia), triglyceride kit (supplier PT. Rajawali Nusindo, Jakarta, Indonesia), isotropenol, liquid nitrogen, Bradford reagent, PBS solution, 1% skimmed milk, Tween 20, 1% Triton X-100, 0.1% SDS, 0.5 and 20 mM EDTA, 1 mM PMSE, 100 and 150 mM NaCl, 1 M NaOH, 5 mM MgCl₂, 10 mM Tris-HCl (pH 7.5), 100 mM Tris-HCl (pH 9.5), standard BSA solution, 50 mg/mL BCIP solution, 50 mg/mL NBT solution, DMF solution, MPO (Bioss-USA) polyclonal primer antibody and anti-rabbit IgG (whole molecule)–alkaline phosphatase-conjugated (Sigma-Aldrich, St. Louis, MO, USA) secondary antibody.

Test Animals. Ethical approval was obtained from the Ethics Committee for Animal Research, Bandung Institute of Technology, with regard to protection and welfare of the animals used in this study (No. 05/KEPHP-ITB/08-2016). The test animals were 22 male Wistar rats aged 10–12 weeks and weighing 200–350 g. The animals were obtained from the Animal Laboratory of School of Pharmacy, Bandung Institute of Technology. The test animals were placed in cages under suitable environments based on animal testing standards.

Grouping of Test Animals. The test animals were categorised into four groups with each group consisting of five to six rats. Group one was the normal group that was not provided any treatment, except normal feeding. Group two was another normal group but with the induction of MI the day before the isolation of the aorta and the heart. Group three consisted of hypercholesterolaemic rats that were fed with food containing high cholesterol and cholic acid, pure cholesterol and PTU. Group four was the hypercholesterolaemia–MI (H-MI) group, in which the rats underwent the induction of hypercholesterolaemia through the administration of high cholesterol as well as the induction of MI the day before the isolation of the aorta and the heart.

Preparation of High-Cholesterol Feed. Hypercholesterolaemia was induced in the animals through the oral administration of a high-cholesterol diet and water ad libitum, as well as PTU, pure cholesterol and cholic acid. PTU 12.5 mg/kg BW/day was suspended in 0.3% CMC-Na with aquadest as the solvent. Cholic acid 200 mg/kg BW/day and pure cholesterol 100 mg/kg BW/day were dissolved in vegetable oil. All the three substances were administered to the rats for 5 consecutive days each week. The high-cholesterol feed composition was prepared from 20 chicken livers, 1 kg cow fat, 1 kg goat fat, 75 quail eggs, 20 duck eggs, 3 kg wheat flour and 2 kg corn flour. Cholesterol-rich feed was administered for 85 days.

MI Animal Model. MI was induced in the animals through the intraperitoneal administration of isoproterenol 150 mg/kg body weight in saline solution a day before the animals were sacrificed.

Collection of Rat Blood. Rat blood was collected for the evaluation of lipid profile. Lipid profile analysis included the measurement of total cholesterol, triglycerides, HDL and LDL. The lipid profiles of all animals were evaluated at the end of the experiment. Only total cholesterol was measured before induction, as well as 1 and 2 months after induction. Rat blood was collected through lateral venous blood vessels in rat tails, while the rats were positioned inside the restrainer. A total of 0.3 mL of blood was collected into Eppendorf tubes and centrifuged for 5 min at 10,000 rpm. After centrifugation, the serum component was collected and stored at −20 °C.

Quantification of Lipid Markers. The levels of total cholesterol, triglycerides, and HDL were measured in rat serum using commercially available kits according to the manufacturer’s instructions. All kits were purchased from PT. Rajawali Nusindo, Jakarta, Indonesia. LDL cholesterol level was determined using the Friedewald equation as follows:

\[
LDL = \frac{Total\ Cholesterol - HDL - (Triglyceride/5)}{150}
\]
Determination of Plasma Atherogenic Index. The plasma atherogenic index is a logarithm of molar concentration ratio (mmol/L) of plasma triglycerides to HDL used to predict the cardiovascular risk. The following equation was applied to determine the plasma atherogenic index:

\[ \text{Plasma atherogenic index} = \log \left( \frac{\text{Triglyceride}}{\text{HDL}} \right) \]

Isolation of Aorta and Heart. The test animals were first sacrificed under CO₂ gas. The aorta and the heart of the animals were isolated in a cold state as soon as possible to prevent protein degradation by protease. The isolated aorta and the heart were placed at the base of an Eppendorf tube for later immersion in liquid nitrogen.

Protein Identification Using Dot Blot. Into every 5 mg of frozen aorta and heart, 300 µL of lysis buffer was added. The lysis buffer consisted of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.1% SDS and 1 mM PMSF. The sample was then homogenised using a sonicator for 5 min with a 30-s on–off cycle. Samples were centrifuged at 10,000 rpm at 4 °C for 5 min. The supernatant containing the total protein was collected and stored at −20 °C.

Characterisation of MPO Proteins. Total protein samples from the aorta and the heart, as much as 4 µg each, were blotted on a nitrocellulose membrane. The nitrocellulose membrane was then immersed in a blocking solution (5% skimmed milk and 0.1% Tween 20 in PBS) for 30 min under shaking at room temperature (25 °C) at low speed (75 rpm). Then, primary MPO antibodies (1:1000) were added to the blocking solution and incubated for 3 h in the shaking incubator. After incubation, the nitro-cellulose membrane was cleansed three times using TBS-T solution, wherein the third cleansing was done for 5 min in the shaking incubator. The TBS-T solution consisted of 10 mM Tris-HCl, 150 mM NaCl, aquadest, and 0.1% Tween 20. The nitrocellulose membrane was then incubated with a secondary antibody in the blocking solution (1:4000) for 1 h in the shaking incubator. Then, the membrane was cleansed in the same manner. Membrane visualisation was performed using NBT–BCIP in an alkaline phosphatase buffer and protected from light. NBT 3.3 mg was dissolved in 46.2 µL DMF and 19.8 µL aquadest. BCIP 1.65 mg was dissolved in 33 µL DMF. The staining reaction was stopped by adding 20 mM EDTA in TBS. The ImageJ V.1.43u software was used for analysing the dot blot results.

Statistical Analysis. The results of the experiments were analysed statistically using SPSS 21.0. Significant differences between the control and the experimental groups were assessed by ANOVA. \( p < 0.05 \) was considered to be statistically significant.

Results

Lipid Profile of Hypercholesterolaemic and MI Rats. At the beginning of induction of hypercholesterolaemia, the average total cholesterol level of all the test groups was 73.36 mg/dL. The mean value was not much different from the average total cholesterol levels in each group, as shown in Figure 1. The statistical analysis showed that the total cholesterol level of the normal group was not significantly different from the total cholesterol levels at all time points of observation. A similar result was observed for the total cholesterol level of the normal–MI group (Figure 1). However, the total cholesterol level of the hypercholesterolaemic and the hypercholesterolaemic–MI groups increased significantly compared to those in the normal and the normal–MI group, respectively. The increase in total cholesterol levels of the hypercholesterolaemic and the hypercholesterolaemic–MI groups was observed until 3 months of induction (Figure 1).

Regarding the levels of triglycerides and HDL, there was no significant difference among all the test groups \( (p > 0.05) \) compared with the normal group, as shown in Figures 2 and 3, respectively. These data indicate that the inductions had not affected the levels of triglycerides and HDL of the test animals till 3 months. However, the LDL levels were significantly increased in the hypercholesterolaemic and the hypercholesterolaemic–MI groups compared to those in the normal group (Figure 4). Regarding the atherogenic index of each group, both the hypercholesterolaemic and the hypercholesterolaemic–MI groups showed a significant increase compared to that in the normal group (Figure 5). This finding suggests that the rat groups in which hypercholesterolaemia was induced had a high risk for cardiovascular disease compared with the rats without hypercholesterolaemia induction.
MPO Levels in the Aorta and the Heart of Hypercholesterolaemic and MI Rats. In the present study, the MPO level was further determined in the aorta (Figure 6) and the heart (Figure 7) of the rat models. It was observed that high cholesterol induction could significantly increase the MPO level in the aorta (Figure 6), but not in the heart (Figure 7), of rats compared to that in the normal group. Concomitant hypercholesterolaemia and MI led to a higher MPO level compared to that with hypercholesterolaemia alone in the aorta and the heart of both rat models.
inhibiting cholesterol conversion to bile acids. PTU works by inhibiting LDL receptors, so that the circulating LDL level in the blood increases. PTU may further decrease the expression of LDL receptors, which results in the atherosclerosis and thus can remove cholesterol from cells, inhibit LDL oxidation, promote endothelial repair, improve endothelial function, possesses anti-thrombotic and anti-inflammatory tendencies and inhibits monocyte attachment to the endothelium.

The association between the risk of coronary artery disease, high levels of LDL cholesterol and low HDL cholesterol levels has been widely investigated. High triglyceride levels are known to be associated with the increasing incidence of cardiovascular disease and the increasing amount of small dense LDL. The ratio between triglycerides and HDL has been used as a strong predictor for MI. In the present study, high cholesterol administration increased the total cholesterol and LDL levels and had no effect on triglyceride and HDL levels, suggesting that the induction for increasing the risk of coronary artery disease was successful.

It is known that a high cholesterol level induces inflammation in the adipose tissue and the vasculature, however, its effect on the vasculature is unknown. The present study showed that MI induces systemic inflammation in both the heart and the vasculature. Concomitant hypercholesterolaemia and MI accelerates inflammation, which will accelerate atheroma plaque formation.

This study implies a pharmacological knowledge of MI contribution to the inflammatory status of the aorta and the heart of hypercholesterolaemic rats. A limitation of this study is that the inflammatory condition was determined by measuring the MPO level using the dot blot method. In the future, MPO protein level must be measured using the western blot method. However, the dot blot results can be used for predicting the western blot results. Furthermore, a histopathological analysis of the aorta and the heart is required to determine the effect of high cholesterol level and/or isoproterenol administration on the aorta and the heart tissue. Furthermore, since this study was conducted on rats, it is unknown whether the results can be extrapolated to humans.

**Conclusions**

This study demonstrated that high cholesterol induction in male Wistar rats significantly affected the expression of MPO in the aorta, and the presence of the MI condition accelerated the inflammation in the aorta. Meanwhile, the presence of the MI condition in the heart of male Wistar rats significantly affected the expression of MPO and was aggravated by the presence of high cholesterol levels.
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Conflict of Interest Statement

The author(s) declare that they have no competing interests.

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