INTRODUCTION
Moderate-intensity exercise induces transient oxidative stress, stimulates redox-sensitive signals, and then increases the antioxidant defense system, leading to cytoprotection. However, heavy exercise induces excessive oxidant stress, resulting in tissue injury. The increase of myeloperoxidase (MPO), an oxidant stress marker belonging to the heme peroxidase superfamily, has been reported as a result of acute heavy exercise in healthy subjects (Suzuki et al., 1996; Nakajima et al., 2010a; Reihmane et al., 2012). MPO is found in myeloid cells, particularly in neutrophils (Ikitimur and Karadag, 2000; Tavora et al., 2009; Nakajima et al., 2010a), and plays an important role in the host defense against bacteria and viruses. On the other hand, it has been suggested to have a role in atherosclerosis through its strong oxidative capacity. MPO-mediated formation of reactive oxidants is enhanced after exhaustive exercise. Low-intensity resistance exercise (LIRE) under the conditions of restricting muscle blood flow (KAATSU) has been reported to strengthen muscles, in a way similar to high-intensity resistance exercise. We investigated the effects of LIRE-KAATSU on neutrophil intracellular MPO and pentraxin 3 (PTX3), an inflammatory marker. Five control subjects and six patients with ischemic heart diseases (IHD) (pPCI 5, pCABG 1) have participated in this study. Each subject performed LIRE (20% 1RM leg extension, 4 sets, 30-15-15-15) under the conditions with or without KAATSU. Neutrophils were immunostained with a specific antibody to PTX3 and MPO and the density of these markers was measured by flow cytometry analysis (FACS). FACS and immunostaining studies showed the existence of PTX3 and MPO in neutrophils. LIRE did not particularly release PTX3 and MPO from neutrophils in healthy subjects under the conditions with or without KAATSU. The similar results were obtained in patients with IHD. These results suggest that the addition of KAATSU to LIRE did not enhance PTX3 and MPO release from neutrophils even in patients with IHD, which may be related to the safety of KAATSU, compared to high-intensity resistance exercises.

Key words: KAATSU training, Myeloperoxidase; Pentraxin 3; neutrophils; resistance exercise
2006; Nakajima et al., 2010). We have previously reported that KAATSU training using 20-30% 1RM resistance exercises increase muscle strength and muscle mass in patients with cardiovascular diseases (Nakajima et al., 2010) and applying blood flow restriction does not affect exercise-induced hemostatic and inflammatory responses of CRP (Madarame et al., 2012). However, responses to KAATSU including neutrophils have not been investigated.

Therefore, the purpose of this study is to investigate the effects of low-intensity resistance exercise with KAATSU on neutrophil intracellular MPO and PTX3 conducted on patients with ischemic heart diseases (IHD) in comparison to control subjects. Results have been derived by assessing the PTX3 and MPO levels in neutrophils isolated from the peripheral blood by using flow cytometry analysis as a result of the exercises.

**MATERIALS AND METHODS**

**Subjects**

Five male control subjects (37 ± 5 years) and six male patients with IHD (post-coronary artery bypass grafting (p-CABG) 1, post-percutaneous coronary intervention (p-PCI) 5, 60 ± 3 years old) participated in this study. These patients had no organic stenosis after CABG or PCI and had no symptoms at the start of this study. This study was approved by the Ethics Committee of the University of Tokyo. All subjects were informed of the methods, procedures and risks, and signed an informed consent document before participation.

**Exercise Protocol**

Each subject performed low-intensity leg extension resistance exercise (20% 1RM, 4 sets) under the conditions either with or without blood flow restriction – the order of which was counterbalanced across subjects. In each exercise session, one set with 30 repetitions was followed by 3 sets with 15 repetitions. A rest period of 1 min was taken between sets. We measured 1RM voluntary force before the exercise.

**Femoral Muscle Blood Flow Reduction Method by KAATSU**

The method for inducing the reduction of muscle blood flow is similar to that described in previous papers (Takarada et al. 2000a; b; Takarada et al., 2002; Takano et al., 2005; Abe et al. 2006; Fujita et al., 2007; Iida et al., 2007; Nakajima et al., 2008). A specially-designed KAATSU belt applies pressure at the proximal ends of both sides of the thighs to restrict venous blood flow. The cuff pressure is set to 200 mmHg.

**Blood Sampling**

Blood samples were obtained using an indwelling heparin-lock catheter inserted into a superficial antebrachial vein on the left arm. The subjects were instructed to sit quietly before taking blood sample for at least 30 min. The baseline (resting/pre-exercise) blood sample was taken before the application of KAATSU or the start of the exercises. Post-exercise blood samples were further obtained within 1-2 min after the exercise and the release of the belt.

**Neutrophils, Monocytes, and Lymphocytes Isolation**

Heparinized whole blood (5 ml) from peripheral blood was layered over 5 ml of Polymorphprep (Axis-Shield, Oslo, Norway) and spun for 35 min at 500 g at 20 °C. Mononuclear cells (MNCs) were harvested from the top band of the sample-medium interface, and polymorphonuclear cells (PMNs) were harvested from the second interface. One ml of 0.45% NaCl solution was added into each fraction to restore normal osmolarity, washed with phosphate-buffered saline (PBS, Nacalai tesque Co., Kyoto, Japan) and spun for 10 min at 270 g at room temperature.

PMNs, mainly composed of neutrophils, were resuspended in a 0.5 ml buffer (3% FBS and 0.05% NaN₃ in PBS), and mixed with a 5 ml ice-cold 70% ethanol for 30 min and spun for 10 min at 270 g at room temperature. Subsequently, PMNs were resuspended in 1 ml 70% ethanol, dropped on a slide glass, and was dried. MNCs, composed of monocytes and lymphocytes, were resuspended in a 1 ml buffer. Then, the cells were performed on a cell sorter (EPICS ALTRA, Beckman Coulter, Fullerton, CA) to sort out monocytes and lymphocytes from the fraction of MNCs. Each sorted portion was spun for 10 min at 270 g at room temperature. Subsequently, monocytes and lymphocytes were resuspended in a 1 ml 70% ethanol, dropped on a slide glass, and dried in a similar manner to PMNs.

**Immunofluorescent Staining**

The fixed cells were washed in PBS, and then blocked for 15 min with 2% horse serum in PBS, and were incubated with the primary antibody in a humid chamber overnight at 4 °C. The primary antibodies used were a rabbit polyclonal anti-human myeloperoxidase (Novus Biological, Littleton, CO) and a rat monoclonal antibody against human PTX3 (clone MNB4, Alexis Biochemicals, San Diego, CA). After the preparation of the antibodies, the cells were washed in PBS and the secondary antibody was incubated for 60 min at room temperature. An Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H+L) antibody (Molecular Probes, Eugene, OR), and a
Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) were used to visualize the MPO and PTX3 expression. For negative controls, normal rabbit IgG and normal rat IgG (Vector laboratories, Burlingame, CA) were used instead of primary antibodies. The nuclei were stained with Hoechst 33258 (Sigma Aldrich). A confocal laser scanning microscope (Olympus Fluoview FV300, Olympus Co., Tokyo, Japan) was used to examine the specimens.

Flow Cytometry Analysis

For the flow cytometry analysis (FACS) using neutrophils as previously reported (Nakajima et al., 2010a), whole blood (300 μl) was lysed with a Versalyse reagent (Beckman Coulter, Fullerton, CA) and spun for 5 min at 270 g. Then, cells were resuspended in a 100 μl of 4% paraformaldehyde for 15 min. Subsequently, 1 ml of the buffer (see above) was added and spun for 5 min at 270 g The cells were resuspended in a 100 μl buffer containing 0.1% saponin for 5 min and incubated for 30 min at room temperature with a mixture of primary antibody (the rabbit polyclonal anti-human myeloperoxidase (Novus Biological, Littleton, CO) and rat monoclonal antibody against human PTX3 (clone MNB1, Alexis Biochemicals, San Diego, CA). Then, the cells were added with a 1 ml buffer containing 0.1% saponin, spun at 270 g and incubated with a secondary antibody (Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H+L) antibody (Molecular Probes Inc., Eugene, OR) and a PE-conjugated Goat anti-rat IgG (H+L) antibody (Beckman Coulter, Fullerton, CA). Finally, the cells were again, resuspended in the buffer. Flow cytometry (FACS) was performed on a flow cytometer (EPICS XL-MCL, Beckman Coulter, Fullerton, CA).

Figure 1 shows a typical example of PTX3 in neutrophils by FACS. The expression of PTX3 was identified in neutrophils. The right side shows neutrophils counterstained with Hoechst33258 which helps visualize the nuclei. The mean fluorescence intensity of PTX3 and MPO from blood taken immediately after the exercise and taken before the exercise was compared. Assuming the mean fluorescence density of MPO and PTX3 in neutrophils isolated from pre-exercise blood is 100%, the relative value of the mean fluorescence density of neutrophils isolated from blood immediately after the exercises is calculated.

Data Analysis

All values are expressed as means ± S.E.M. Student’s pared t test was used to compare two sets of data from the same subjects. Differences were considered significant if p values were less than 0.05.

RESULTS

First, we investigated the existence of MPO and PTX3 in human neutrophils. Immunostaining studies demonstrated the presence of MPO (Fig. 2A) and PTX3 (Fig. 2B) in human neutrophils taken from control subjects. Flow cytometry analysis also showed the existence of both PTX3 (Fig. 1) and MPO (Fig. 5) using a specific antibody. The data were obtained from a control subject (Fig. 1) and a patient with IHD (Fig. 5), respectively. The expression of MPO and PTX was also separately investigated in neutrophils, monocytes, and lymphocytes. As shown in Fig. 3, the expression of MPO was particularly observed in neutrophils (A), and no expression was detected in negative controls without the antibody (B). It was also observed to a lesser extent in

![Flow cytometry analysis of PTX3 expression in human neutrophils isolated from peripheral blood (see methods). The neutrophils were counterstained with Hoechst33258 to visualize nuclei shown on right.](image-url)
Figure 2. Immunostaining of MPO (A) and PTX3 (B) in neutrophils.

Figure 3. Immunostaining of MPO in neutrophil, monocytes, and lymphocytes. A: neutrophil B: negative control (without antibody) C: monocytes D: lymphocytes. Note that the expression of MPO in healthy subjects is particularly observed in neutrophils, and to a lesser extent in monocytes.
monocytes (C). The very weak staining was observed in human lymphocytes (D). The marked expression of PTX3 was observed in neutrophils (A) only as shown in Fig. 4. No expression was detected in negative controls without the antibody (B). The definite expression of PTX3 was not observed in human monocytes (C) and lymphocytes (D), compared with neutrophils (A).

Secondly, we investigated the effects of 20% 1RM low-intensity resistance exercise with or without the restriction of muscle blood flow (KAATSU) on neutrophil MPO and PTX3, by using FACS. The typical data under the KAATSU condition obtained from a 67-year old patient are shown in Fig. 5. The mean fluorescence density of MPO in neutrophils isolated from post-exercise blood was similar to that obtained at rest (pre-exercise blood).

Five control male subjects and 6 patients with IHD were also examined as shown in Figs. 6 and 7. Neutrophils isolated from blood immediately after exercise without KAATSU exhibited no significant differences in the mean fluorescence for MPO (Fig. 6).
KAATSU training and neutrophil PTX3/MPO

and PTX3 (Fig. 7) compared to that isolated from pre-exercise blood in control subjects (Figs. 6 & 7, left side) and patients with IHD (Figs. 6 & 7, right side). Similar results were obtained from patients with IHD under the conditions with KAATSU (Figs. 6 & 7). Neutrophils isolated from blood immediately after exercise with KAATSU also exhibited no significant changes in the mean fluorescence for MPO (Fig. 6) and PTX3 (Fig. 7) from control subjects and patients with IHD.

DISCUSSION

The major findings of the present study are as follows; 1) FACS and immunostaining studies have shown the existence of PTX3 and MPO in neutrophils. 2) 20% 1RM low-intensity resistance exercise did not significantly release PTX3 and MPO from neutrophils in control subjects under the conditions with or without KAATSU. Similar results have been observed in patients with IHD. Thus, the addition of muscle blood flow (KAATSU) restrictions to low-intensity resistance exercise did not enhance PTX3 and MPO release from neutrophils in patients with IHD, which may be related to the safety of KAATSU, compared to high-intensity resistance exercises.

First, the present study using FACS and immunostaining studies has shown the existence of MPO and PTX3 in neutrophils, which is consistent with the previous papers (Ikitimur and Karadag, 2000; Jaillon et al., 2007; Tavora et al., 2009; Nakajima et al., 2010a). As shown in Fig. 3, the expression of MPO was particularly observed in neutrophils and to a lesser extent in monocytes. Also, the expression of PTX3 has only been observed in neutrophils as shown in Fig. 4. Therefore, we have investigated the effects of low-intensity resistance exercise on intracellular MPO and PTX3 in neutrophils using flow cytometry analysis. Similarly, changes in neutrophil surface receptor expressions including CD16 and degranulation have been investigated immediately after a high-intensity exercise (a treadmill for 1 h at 80% VO2max) (Peake et al., 2004).

Acute exercise is an important stressor to the body, leading to an activation of immune responses (Hoffman-Goetz and Pedersen, 1994; Pedersen and Hoffman-Goetz, 2000; Peake et al., 2004; 2005a; Buttner et al., 2007) depending on the intensity. The exercise-induced acute phase response consists of typical changes including leukocytosis and release of cytokines and acute phase proteins, similar to acute phase responses evoked by other stressors such as trauma and sepsis (McCarthy et al., 1992; Ostrowski et al., 2000; Shephard, 2001; Yamada et al., 2002; Natale et al., 2003; Peake et al., 2005b; Mooren et al., 2006; Zaldivar et al., 2006). PTX3 and CRP have been shown to be independent high-sensitive markers for inflammation under the various pathophysiological conditions (Presta et al., 2007; Mantovani et al., 2008), and the increase of inflammatory markers such as CRP and PTX3 have been reported in acute heavy exercises (Castell et al., 1997; Nakajima et al., 2010a). High-intensity exercises also increase serum MPO concentration which depends on the exercise intensity (Peake et al., 2004; Reihmane et al., 2012).
Recently, we have shown that neutrophils isolated from blood immediately after high-intensity resistance exercise (70% IRM) releases MPO and PTX3 in control subjects using flow cytometry analysis (Nakajima et al., 2010a). However, the present study showed that 20% IRM low-intensity resistance exercise did not significantly release neutrophil MPO and PTX3 in control subjects. Thus, the exercise-induced neutrophil MPO and PTX3 release appears to depend on exercise-intensity. The present study also investigated the responses to KAATSU in healthy subjects. The application of KAATSU did not enhance neutrophil MPO and PTX3 significantly in healthy subjects. These results were in contrast with the results from high-intensity exercise, but somewhat consistent with the previous papers showing that applying blood flow restriction does not affect exercise-induced inflammatory responses of CRP, another inflammatory marker (Madarame et al., 2012).

MPO has a role in atherosclerosis through its strong oxidative capacity. MPO-mediated formation of highly reactive oxidants, hypochlorous acid (HClO), causes consumption of nitric oxide (NO) on endothelial cells and the oxidation of LDL on vascular wall in atherosclerotic regions. The excessive formation of MPO leads to the formation of atheroma, then plaque rupture, and acute coronary syndrome as well (Tavora et al., 2009). Thus, MPO is a key component in the development and progression of atherosclerosis and other forms of cardiovascular diseases (Ikitimur and Karadag, 2010). Therefore, we also investigated the effects of low-intensity resistance exercise with or without KAATSU on neutrophil MPO and PTX3 in patients with cardiovascular diseases as well as healthy subjects. The present study has shown that KAATSU training using low-intensity exercise did not significantly release neutrophil MPO and PTX3 release in these patients. Similarly, we have recently reported that applying blood flow restriction did not enhance exercise-induced inflammatory responses of CRP in patients with IHD (Madarame et al., 2012). Thus, the addition of KAATSU to low-intensity resistance exercise did not enhance inflammatory responses (CRP and PTX3) and neutrophil MPO release from neutrophils even in patients with IHD, which may be related to the safety of KAATSU compared to high-intensity resistance exercise. In fact, we have reported that KAATSU resistance training using 20-30% IRM resistance exercises can increase muscle strength and muscle mass in patients with cardiovascular diseases. Furthermore, no obvious side effects including coronary events have occurred (Nakajima et al., 2010b). However, since the patients used in this study are very stable, and have normal cardiac function and no significant coronary artery stenosis, who have received PCI or CABG. Further studies using relatively high-risk patients or cardiac dysfunction are needed to justify this.

In conclusion, the addition of KAATSU to low-intensity resistance exercise did not enhance PTX3 and MPO release from neutrophils in patients with cardiovascular diseases, which suggests the safety of KAATSU in cardiac rehabilitation compared to high-intensity resistance exercise.

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