Biochemical and Biophysical Research Communications 405 (2011) 58–63

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NO signaling in exercise training-induced anti-apoptotic effects in human neutrophils

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ARTICLE INFO

Article history:
Received 18 December 2010
Available online 31 December 2010

Keywords:
Exercise training
Neutrophil
Apoptosis
Nitric oxide
Mcl-1

ABSTRACT

Short-lived neutrophils play a predominant role in innate immunity, the effects of exercise training on neutrophil survival is unclear. In this study, we investigated the underlying mechanisms of training effects on human neutrophil apoptosis. Healthy male subjects were trained on a cycling ergometer for 8 weeks and followed by 4 weeks of detraining. Blood neutrophils were collected before exercise, after training, and after detraining. Comparing with pre-exercise specimens, neutrophils collected after training showed reduced apoptosis rate, which partially returned after detraining. Various intracellular proteins, including iNOS, Mcl-1, A1, Grp78, and IL-8, were upregulated by training, and they remained high after detraining. Upregulated iNOS was closely correlated with these anti-apoptotic molecules in neutrophils. Furthermore, the possible mechanism by which iNOS suppressed apoptosis was explored. Neutrophil apoptosis was accelerated by blocking and retarded by stimulating the endogenous iNOS activity. As an anti-apoptosis mediator of NO signaling, the Mcl-1 level dropped by depletion of the major NO downstream molecule cGMP and such loss of Mcl-1 was avoidable when supplying exogenous NO. Upon activation of NO-cGMP signaling, neutrophils held increased Mcl-1 expression and delayed apoptosis. Collectively, our results suggested that exercise training may retard neutrophil apoptosis by upregulating the iNOS-NO-cGMP-Mcl-1 pathway.

1. Introduction

Regular exercise under moderate intensity has been associated with numerous health benefits, therefore the promotion of regular exercise for normal people and patients is recommended. Epidemiological reports show that physical conditioning is related to low incidences of various oxidative stress-associated diseases, including heart disease, acquired diabetes, neurodegenerative disorders, and many forms of cancer [1,2]. In parallel, animals with exercise training show fewer incidences of oxidative stress-associated diseases along with prolonged life span [3,4]. The cellular adaptation to exercise training, those include increased expression of inducible nitric oxide synthase (iNOS) and certain anti-oxidative enzymes, is accompanied with reduced apoptosis in skeletal muscle, heart, and brain of aged animals [5–8]. Husain (2003) had demonstrated that some beneficial effects of exercise training are blunted when animals are subjected to chronic NOS inhibition [9]. It seems to be common in various tissues that exercise training may retard stress- or senescence-related cellular apoptosis through the modulation of NO signaling and oxidant scavengers.

Neutrophils are the most abundant population of circulating leukocytes, about 60% of the hematopoietic capacity of normal human bone marrow is used for production of neutrophils [10]. Postmitotic neutrophils are naturally short-lived (with a life span in the blood shorter than 24 h), they gradually loss L-selectin and undergo spontaneous apoptosis as they aging in the circulation [11]. Neutrophils play a major role in the early defense against microbial infections, however they become poorly responsive to challenges upon initiation of apoptosis [12]. Quiescent neutrophils can generate considerable amounts of NO, similar to the endothelium-derived NO, through their constitutively expressed nNOS and iNOS [13–15]. Interestingly, neutrophil iNOS transcripts and proteins of subjects are upregulated in response to acute running [16]. If neutrophils express extra amounts of iNOS protein, that may result in the systemic NO rise. Elevated levels of nitrite/nitrate (NO_2^-/NO_3^-; stable metabolites of NO) and cyclic GMP (major downstream component of NO pathway) are also found in the plasma and/or

Abbreviations: FasL, Fas ligand; GM-CSF, granulocyte/macrophage colony-stimulating factor; Grp, glucose-regulated protein; HSP, heat shock protein; IL, interleukin; MB, methylene blue; MFI, mean of fluorescence intensity; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TRAIL, TNF-related apoptosis inducing ligand; VO_2max, maximal oxygen consumption.

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doi:10.1016/j.bbrc.2010.12.123

the urine of athletes or regularly exercised subjects [17–19]. Regarding host immunity, the evoked NO surge from neutrophils serves as an effector to kill infectious organisms, the role of physiological NO in neutrophil apoptosis is another important issue and remains unclear.

The fate of a cell depends on its intracellular ratio of pro-apoptotic and anti-apoptotic force. Both pro- and anti-apoptotic properties of NO have been reported. On one hand, large amounts of NO increase the formation of peroxynitrite ($ONOO^-$) which causes DNA damage and consequently results in apoptosis in general cells [20], and on the other hand, limited amounts of NO exert anti-apoptotic effects against pro-apoptotic stimulation in isolated neurons and lymphocytes [21,22]. The mitochondrion is a key organelle in controlling apoptosis; its membrane depolarization causes the release of pro-apoptotic molecules. NO in physiological concentrations (nanomolar range) reversibly inhibits cytochrome c oxidase (complex IV in the respiration chain), which leads to mitochondrial membrane hyperpolarization and thus prevents apoptosis [23]. Various anti-apoptotic molecules are directly or indirectly upregulated by NO. Mcl-1 and A1, the major anti-apoptotic Bcl-2 family members present in newborn human neutrophils, are localized to mitochondria to protect them against pro-apoptotic insults [24]. Moreover, the expression of Mcl-1 and A1 in neutrophils is augmented by IL-8, which is induced by pure NO donors but inhibited by peroxynitrite [25,26]. The glucose-regulated protein 78 (Grp78), a member of the HSP family, can enhance the cellular resistance to apoptosis by acting as a chaperone in maintaining the membrane integrity of mitochondria and endoplasmic reticulum. The expression of Grp78 is highly upregulated in iNOS-inducible cells while they are stimulated to generate 150–200 nM NO [27]. As the anti-apoptotic molecules in neutrophils are relatively labile, their level would determine the outcome of neutrophils. The aims of this study were to investigate the effects of exercise training on neutrophil apoptosis, and the possible role of NO signaling pathway in mediating such effects. In this study, we demonstrated that exercise training retarded neutrophil apoptosis by enhancing iNOS and certain NO-regulated anti-apoptotic molecules.

2. Materials and methods

2.1. Reagents

Antibodies against Mcl-1, iNOS, and Grp78 were purchased from PharMingen (San Diego, CA, USA); anti-A1 antibody was from Cell Signaling Tech (Beverly, MA, USA); anti-interleukin 8 (IL-8) antibody was from BioLegend (San Diego, CA, USA); antibody against TNF-related apoptosis inducing ligand (TRAIL), Fas and Fas ligand (FasL) were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Lipopolysaccharide (LPS), sodium nitroprusside (SNP) and other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Subjects and experimental design

Six healthy, sedentary male volunteers between 21 and 27 years of age were recruited. The subjects were 23 ± 1 (mean ± SE) years old, 170 ± 2 cm in height, and of 66.8 ± 4.2 kg body weight. None of the participants was taking medications in the period of this study. Maximal oxygen consumption (VO_2max) was measured during incremental exercise on a leg cycle ergometer until exhaustion. The subjects were trained by an 8 weeks exercise program at moderate intensity (70% of their individual VO_2max) for 30 min per day, 5 days per week. They were then detrained for 4 weeks. Our training program was approved by the Human Ethics Committee of National Cheng Kung University Medical College. Peripheral blood samples were collected before and after (2 days after the last cycling to avoid the acute effects of exercise) the exercise training, and the end of the 4th week of detraining. Total and differential leukocyte counts, numerous molecule expressions in mononuclear or polymorphonuclear leukocytes, and spontaneous apoptosis of purified neutrophils were determined in the same batch.

2.3. Determination of neutrophil apoptosis

Neutrophils were isolated by using a double gradient formed by layering Histopaque-1077 over Histopaque-1119, and the residual erythrocytes were removed by hypotonic lysis. The purity of isolated neutrophils was >95%; cell viability immediately after purification was examined by trypan blue exclusion and was greater than 98%. To determine the spontaneous apoptosis rate, neutrophils were resuspended at 3×10^6 cells/ml of RPMI 1640 supplemented with 5% FCS, and were cultured for various time periods in an incubator at 37 °C with 5% CO_2. Apoptotic neutrophil was determined by its annexin V-FITC labeling and nuclear condensation. The percent of apoptotic neutrophils was verified by flow cytometric measurements of the cells labeled with annexin V-FITC on their outer plasma membrane. Nuclear morphologic changes of apoptotic neutrophils from polymorphic to condensed and rounded shapes were further confirmed. To examine the nuclear morphology, cells were centrifuged via cytospin at various time points during incubation, fixed with cold methanol, stained by Wright's staining procedure, and observed using a 100× oil objective lens. At least 500 cells per specimen were counted.

2.4. Determination of various molecule expressions

The expressions of specific molecules on neutrophils collected at different time points were assessed simultaneously to diminish the unavoidable bias. The surface molecules (including Fas, FasL, and TRAIL) and intracellular molecules (including iNOS, Mcl-1, A1, Grp78, and IL-8) were determined. Briefly, neutrophils were fixed with 4% paraformaldehyde immediately after purification and subsequently incubated with various primary antibodies (3 μg/ml) containing 1% BSA at 4 °C overnight for the detection of cell surface molecules. To detect the intracellular molecules, cells were pretreated with 0.5% Triton X-100 for 30 min and incubated with a primary antibody in PBS containing 2% BSA at 4 °C overnight. After cold buffer washes, cells were further incubated for 1 h with fluorophore-conjugated secondary antibody, and analyzed by flow cytometry within an hour. The relative expression of specific molecule was presented as mean of fluorescence intensity (MFI).

2.5. Statistics

All data were presented as mean ± SE. Statistical analysis was performed by Student's *t*-test, except the time-course results of neutrophil apoptosis which were analyzed by one-way repeated measures ANOVA. Correlation statistics (including the Pearson's correlation coefficient *r* and the *p* value) were obtained from simple regression analysis. Results were considered to be significantly different when $p < 0.05$.

3. Results and discussion

3.1. Effects of exercise training and detraining on neutrophil apoptosis and iNOS expression

According to the measurement of cardio-pulmonary capacity, the physical conditions of all subjects were functionally adjusted by the training and detraining regime. After 8 weeks of exercise

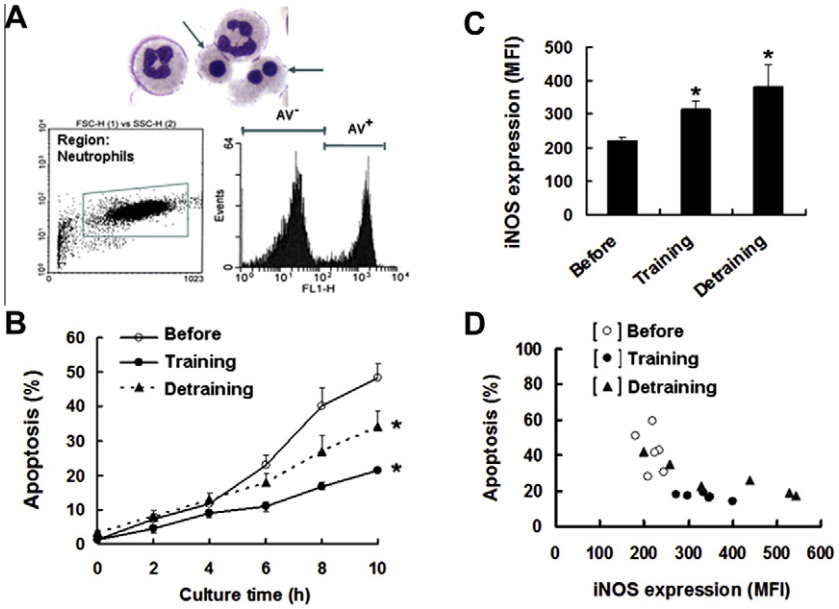

Fig. 1. Effects of exercise training and detraining on neutrophil apoptosis and iNOS expression. (A) Neutrophil apoptosis could be identified and quantified by morphologic changes and flow cytometry. Arrows mark the apoptotic neutrophils, which show relatively high annexin V fluorescence. (B) The rate of spontaneous apoptosis was ascertained by monitoring the fractional percentage of annexin V-positive neutrophils present in culture at different time points. (C) The iNOS level was determined in freshly isolated specimens and presented as mean of fluorescence intensity (MFI). (D) A negative correlation can be clearly observed between apoptosis percentage at 10 h culture time and their iNOS expression. Neutrophils with iNOS level higher than 300 MFI showed minimal apoptosis. $^{*}p < 0.05$, compared with before exercise.

training, VO_2max of the subjects was improved by an average of 28% ($p < 0.05$, from 29.3 ± 1.5 to 37.5 ± 3.2 ml·kg^{-1}·min^{-1}). Their resting heart rates were significantly reduced to 68 ± 2 min^{-1} after training when compared to before exercise (75 ± 3 min^{-1}), and were returned to pre-exercise values after the 4 weeks detraining (74 ± 3 min^{-1}). The circulating leukocyte composition of the resting subjects after training and detraining remained same as before exercise (Supplementary materials, Table S1). Although the neutrophils composed near 60% of leukocytes in all blood samples, their spontaneous apoptosis were retarded by exercise training (Fig. 1A and B). The training effect on neutrophil apoptosis was partially reversed after detraining. In addition, training caused higher iNOS expression in neutrophils (Fig. 1C), but not in lymphocytes and monocytes (data not shown), and such elevation was lasting to the 4th week of detraining. Interestingly, we found a negative correlation between neutrophil iNOS expression and their apoptosis (Fig. 1D). The iNOS likely plays a role in controlling the spontaneous apoptosis of blood neutrophils.

The effect of exercise training should be caused by the accumulative action of individual exercise. Body temperature, cellular oxidative stress, and certain cytokines/hormones are transiently rising during every exercise session. Systemical elevation of particular factors, such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and glucocorticoids, unavoidably affect blood neutrophils. GM-CSF and glucocorticoid can synergistically suppress neutrophil apoptosis by increasing the stability of Mcl-1 and blocking the pro-apoptotic effect of reactive oxygen species (ROS), respectively [28–30]. However, the transient increases in plasma GM-CSF and glucocorticoid hardly explain the lasting anti-apoptosis effect of exercise training up to 4th week of detraining. Therefore, the persistent elevation of intracellular iNOS protein may play a crucial role in the training-exerted anti-apoptosis. Although how exercise training increases neutrophil iNOS expression needs to be clarified, the oxidative challenge-induced adaptive response to repeated exercise may be a possible mechanism [31]. One may argue that how short-live neutrophils memorize such adaptations, it is plausible that any long-lasting effect of exercise on neutrophils is inherited from their progenitor cells.

3.2. Long-term upregulation of various anti-apoptosis molecules by exercise training

Like iNOS, various anti-apoptotic molecules, including Mcl-1, A1, Grp78, and IL-8, were upregulated by exercise training and they remained at high levels after detraining (Fig. 2A). Furthermore, the levels of these anti-apoptotic molecules were positively correlated with that of iNOS ($p < 0.0001$; Fig. 2B). Among them, Mcl-1 has the superlative correlation with iNOS ($r = 0.92$). In contrast, training did not alter the expression of several extrinsic apoptosis inducers, including Fas, FasL, and TRAIL (Supplementary materials, Table S2).

Whether a cell survives or undergoes apoptosis is determined by the counteraction between pro- and anti-apoptotic molecules. Apoptosis antagonists in neutrophils should be key targets to be regulated upon training-decreased apoptosis, because these cells naturally express limited anti-apoptotic molecules but constitutively produce the cytotoxic ROS and pro-apoptotic Bcl-2 family proteins (including Bad, Bax, Bak, and Bid) [24]. Previous studies have demonstrated that NO augments the protein content of Grp78 by several folds which in turn protects cells from apoptosis by blocking caspase activation [27,32]. Moreover, in human neutrophils NO elevates the accumulation of IL-8, which delays neutrophil apoptosis by increasing anti-apoptotic molecules (including Mcl-1, A1, and 14-3-3ξ protein) and decreasing pro-apoptotic molecules (such as Bid and TRADD) [25,33]. Mcl-1 and A1 are the major members of anti-apoptotic Bcl-2 family proteins expressed in mature human neutrophils [34,35]. Because Mcl-1 and A1 transcripts are extremely unstable (half-lives = ~3 h), the gradual degradation of Mcl-1 and A1 will result in the depolarization of mitochondrial membrane by Bax insertion and initiate spontaneous apoptosis of neutrophils [24]. Collectively, in high iNOS-expressed neutrophils the greater NO generation may

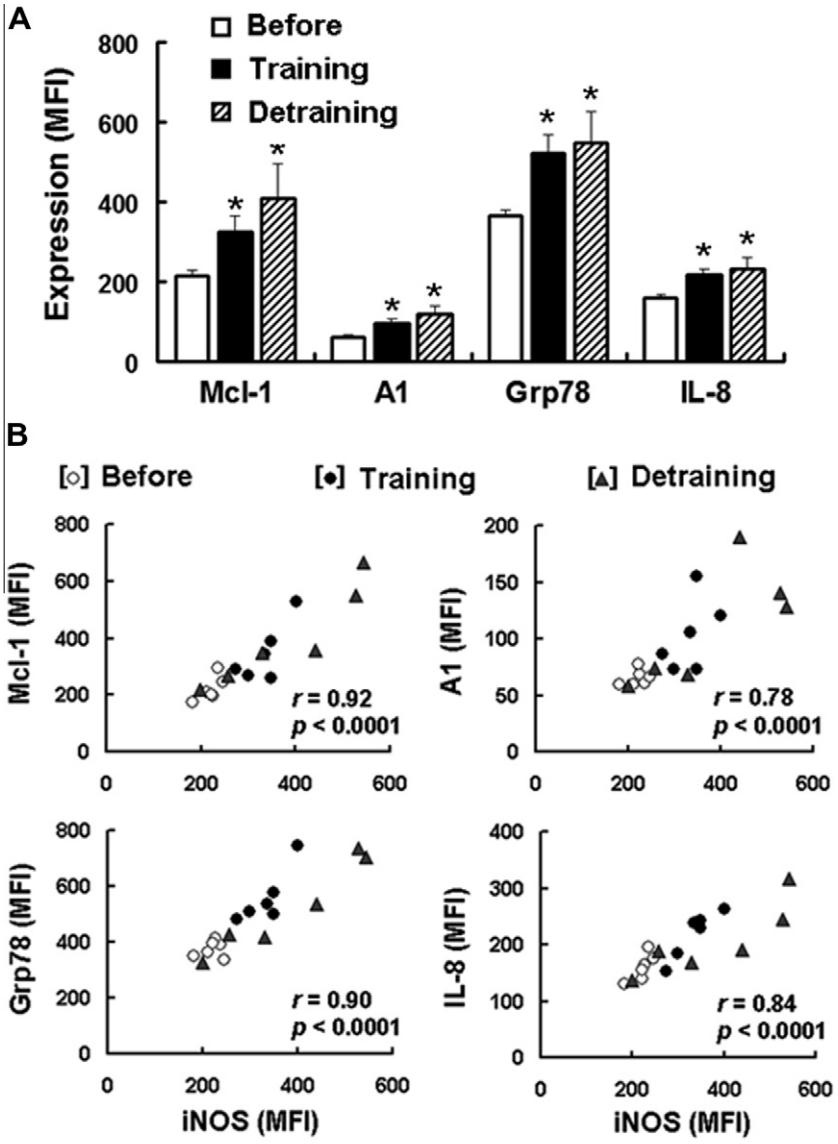

Fig. 2. Effects of exercise training and detraining on the expression of anti-apoptotic molecules. (A) The levels of Mcl-1, A1, Grp78, and IL-8 in freshly isolated neutrophils were determined by flow cytometry (*$p < 0.05$, compared with before exercise; $n = 6$). (B) Positive correlation can be established between the level of iNOS and those of various anti-apoptotic molecules.

contribute to the enhanced Grp78, IL-8, Mcl-1, and A1, and then interrupt the spontaneous apoptosis cascade.

3.3. Regulation of apoptosis by manipulating iNOS and NO signaling

Neutrophils naturally synthesize NO by constitutively expressed iNOS. NO can activate soluble guanylyl cyclase to generate cGMP and subsequently modulates many cGMP effector proteins. Several approaches were taken to examine the role of this NO signaling pathway in neutrophil apoptosis. First, we investigated the effects of iNOS inhibition and induction by aminoguanidine (AG, a selective inhibitor of iNOS) and lipopolysaccharide (LPS, an iNOS stimulator), respectively. Results in Fig. 3 showed that the neutrophil apoptosis was effectively increased by AG and decreased by LPS treatment. Mild dose of LPS (100 ng/ml) was used to stimulate neutrophils of sedentary subjects, as LPS is approved to induce unstimulated neutrophils producing iNOS and NO [36]. AG-augmented apoptosis was abolished by co-incubation with LPS. This indicated that endogenous iNOS/NO did involve in the control of neutrophil apoptosis. Next, in order to verify the NO effect on apoptosis is mediated by cGMP or not, we depleted the NO-dependent intracellular cGMP by using methylene blue (MB), an inhibitor of soluble guanylyl cyclase. The MB treatment caused a significant increase in apoptosis, and that was reversed by LPS. Additionally, an alternative source of NO derived from sodium nitroprusside (SNP) also diminished the MB-evoked neutrophil apoptosis by up to 68% (apoptosis percentage of vehicle-, MB-, and MB/350 μM SNP-treated groups: 39 ± 5%, 89 ± 9%, and 55 ± 9%, respectively; as shown in Fig. 4). Estimated 550 nM of NO was released from 350 μM of SNP in aqueous solution [37]. The inhibitory role of NO-cGMP signaling in neutrophil apoptosis was evident. Furthermore, we determined the correlation between NO signaling and Mcl-1 expression. Early Mcl-1 induction or prevention-of-degradation conceivably retards the apoptosis cascade [35]; therefore, the Mcl-1 level at 4 h and the apoptosis percentage at 10 h of cultured neutrophils were examined (Fig. 4). In the presence of MB,

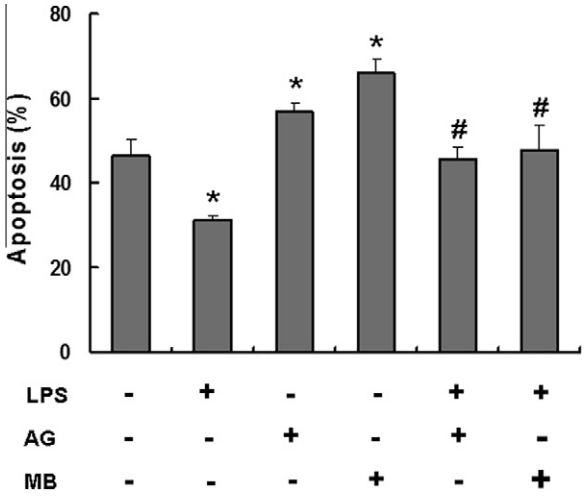

Fig. 3. Effects of iNOS activity on neutrophil apoptosis. Neutrophils isolated from sedentary control subjects were treated with lipopolysaccharide (LPS, 100 ng/ml), aminoguanidine (AG, 30 mM), and methylene blue (MB, 5 μM) for 10 h in culture. $^{*}p < 0.05$, compared with the vehicle; $^{\#}p < 0.05$, compared with single treatment; $n = 4$.

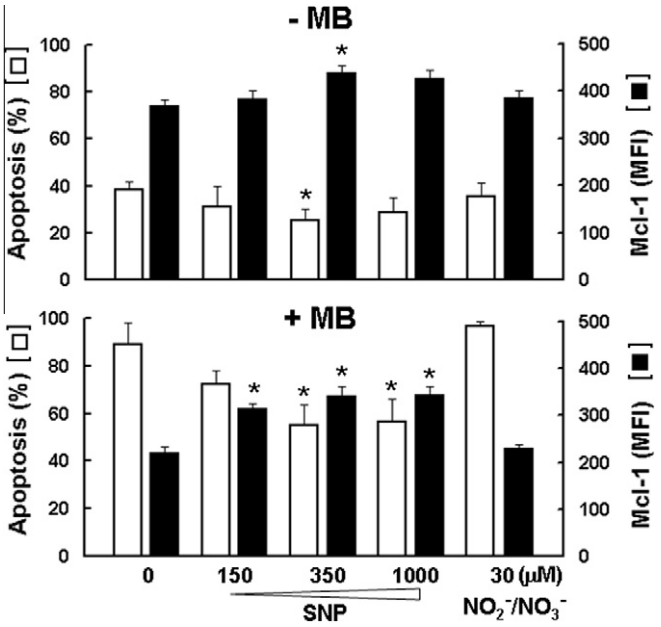

Fig. 4. Role of NO-cGMP signaling in neutrophil apoptosis and Mcl-1 expression. Neutrophils isolated from sedentary control subjects were treated with SNP (a NO donor) or NO metabolites (NO_2^-/NO_3^-), either in the absence or presence of methylene blue (MB, a guanylyl cyclase inhibitor, 5 μM). Apoptosis percentage and Mcl-1 expression level in cultured neutrophils were analyzed by flow cytometry at 10 and 4 h, respectively. $^{*}p < 0.05$, compared with the corresponding vehicle-treated cells; $n = 4$.

neutrophils drastically lost their Mcl-1 protein (a reduction form 368 ± 13 to 219 ± 14 of MFI) and SNP almost reversed such reduction (form 219 ± 14 to 339 ± 23 of MFI). SNP had relatively mild protective effects in the absence of MB. It is suggested that a part of Mcl-1 expression in un-stimulated neutrophils is due to the basal NO-dependent cGMP generation which eliminated by MB and restored by SNP. Finally, we also showed that the major NO metabolites, nitrate NO_3^- and nitrite NO_2^-, 30 μM in total, did not exert any effects on neutrophil Mcl-1 or apoptosis (Fig. 4). Taken together, these results suggest that the iNOS-NO-cGMP signaling pathway plays an important role in maintaining the Mcl-1 level and consequently retarding neutrophil apoptosis.

The NO-cGMP pathway in regulating apoptosis has not been completely clarified. Our results indicated that the constitutive and inducible NO-cGMP signaling may retard neutrophil apoptosis in company with the Mcl-1 upregulation. Mcl-1 is a rapid turnover protein with a short half-life of 2–3 h, prevention of its turnover by inhibiting proteasome has a parallel effect on delaying neutrophil apoptosis [28]. Therefore, upregulation of Mcl-1 plays a crucial role in apoptosis retardation. The NO in physiological concentrations acts as an intracellular regulator of apoptosis in various cells. It has been demonstrated that low concentration (100 μM) of SNP protects chondrocyte death by increasing Bcl-$_{XL}$ and Mcl-1 levels [38], NO also promotes the synthesis of Bcl-2 protein via cGMP formation and results in decreased apoptosis in some primary cultured cells and cell lines [27,39,40]. Many signaling pathways triggered by either NO or cGMP, such as MEK/ERK, p38 MAPK, PI3K/Akt or JAK/STAT3, have been implicated in regulating Mcl-1 expression under various physiological circumstances [41,42]. For these reasons, in neutrophils endogenous NO may upregulate Mcl-1 against apoptosis program through the cGMP-dependent mitogen-activated protein kinase pathways.

In conclusion, exercise training activates the iNOS-NO signaling in neutrophils to antagonize intracellular apoptosis signals via the cGMP-dependent upregulation of anti-apoptotic molecules especially the Mcl-1. In addition to the NO-related long-term regulations, exercise training also activated other short-term regulations that might explain why the apoptosis rate of neutrophils showed partially increased kinetics after detraining. As a whole, to remodel tissue cells into a survival-favorable condition seems a valuable benefit of exercise training.

Acknowledgments

This study was funded by National Science Council, Taiwan (Grant numbers: NSC91-2320-B-273-009 and NSC92-2320-B-273-002), and Tzu Chi University (Grant number: TCMRC-P-95004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.123.

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