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Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity

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Abstract
The number of circulating stem cells with endothelial progenitor capacity (EPCs) inversely correlates with the number of cardiovascular risk factors. In this study we sought to investigate the effects of vascular H_2O_2 on circulating EPC levels. In C57BL/6 mice 3 weeks of freely moving or forced physical activity or voluntary exercise failed to increase circulating EPCs defined as double positive for Flk-1 and CD34, CD133 or Sca-1. Likewise, neither insertion of additional genes encoding for catalase (cat^{++}) or eNOS nor eNOS knock-out changed EPCs in resting mice. In striking contrast, inhibition of catalase by aminotriazole strongly reduced circulating EPCs in sedentary cat^{++} and their transgen-negative littermates (cat^{n}), while forced or voluntary exercise training of cat^{++} mice significantly increased the number of circulating EPCs. The latter effect was completely inhibitable by aminotriazole. These data suggest that endogenous vascular H_2O_2 likely contributes to the impairment of important stem cell-induced vascular repair mechanisms in cardiovascular disease.

Keywords: *Hydrogen peroxide, endothelial progenitor cells, exercise, reactive oxygen species.*

Introduction

Recent evidence suggests that regeneration of damaged vascular endothelium involves the participation of stem cells mobilized from the bone marrow. Asahara et al. [1] demonstrated that circulating $CD34^{+}$-angioblasts within human peripheral blood are able to differentiate *in vitro* to endothelial phenotype. These 'endothelial progenitor cells' contribute to important vascular repair mechanisms such as re-endothelialization and can improve organ blood flow by homing into ischemic regions. Here, EPCs contribute to the formation of entirely new vessels and/or release angiogenic factors such as vascular endothelial growth factor (VEGF) in a paracrine manner [2–4]. These potentially beneficial effects are impaired when the number and/or functional activities of EPCs are reduced.

Clinical trials revealed that the number of circulating EPCs and EPC homing is decreased in subjects with cardiovascular risk factors, such as hypercholesterolaemia, diabetes and smoking [5,6]. All these pathologies are associated with increased vascular oxidative stress and it has been suggested that a lower number of circulating EPCs indicates the progression of cardiovascular damage [7]. Proliferative EPCs showed decreased clonogenic capacity after treatment with oxidants such as H_2O_2 [8] and an increase of reactive oxygen species such as H_2O_2 likely impairs vessel growth [9]. Therefore, reactive oxygen species may directly influence stem cell-induced vascular repair mechanisms by reducing the number of circulating EPCs.

Exercise has been reported to increase the number of circulating EPCs in mice [10]. Furthermore, exercise is an effective anti-oxidative intervention which can prevent acute cardiovascular events and reduce cardiovascular mortality [11]. We hypothesized that the strong vascular oxidant H_2O_2 interferes with

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exercise-induced increases of circulating EPCs. In the present study we investigated the effect of H_2O_2 on the number of circulating EPCs in sedentary mice and in mice subjected to exercise. To accomplish this, we used normal C57Bl/6 mice and mice of a transgenic strain carrying an endothelial-specific overexpression of catalase [12,13].

Material and methods

Experimental animals

We generated a transgenic construct, in which human catalase was inserted between murine Tie-2 promotor (2.1 kb) and a 10 kb Tie-2-enhancer and this construct was used to target catalase gene expression to the vasculature as described previously [13]. Founder mice were crossed 15 times to C57BL/6 mice to generate a C57BL/6 background. Transgene negative littermates (cat^n) served as controls. In some experiments mice deficient for eNOS gene ($eNOS^{-/-}$) and new generated mouse strain with a endothelial-specific over-expression of bovine eNOS driven by the murine Tie-2 promotor were used ($eNOS^{++}$) [13,14]. In addition, C57Bl/6 mice were used to study the effect of different exercise protocols on circulating levels of EPCs.

Sedentary, moving and exercised mice

All mice were bred at the university's animal facilities in an SPF-area. Mice were randomly assigned to one of four groups: (1) sedentary mice living alone for 5 weeks in small cages with a floor space of 0.3 m^2 (Figure 1A), (2) moving mice housed in groups of four-to-five males in cages with a floor space of 0.9 m^2 (moving mice, Figure 1A), (3) forced exercised group (15 days lasting exercise programme on a treadmill, Figure 1B) or (4) voluntary exercised mice (Figure 1C). Mice living in groups were fighting, running and climbing for the most part of their active daily cycle, while singularized mice were predominantly resting during their active daily cycle and showed a low physical activity as evidenced by measuring the heart weight/body weight ratio and skeletal citrate synthase activity [15]. In some experiments soleus weight/body weight and soleus weight/tibia length ratios were calculated as additional exercise performance measures. Citrate synthase activity was measured in soleus muscle as described previously [15].

Exercise protocols

For voluntary training, the mice were housed individually in cages supplied with running wheels (0.25 m in diameter, Tecniplast, Germany) and equipped with counters to record the daily running distance. The mean running distance for C57Bl/6 was 4.65 ± 0.3 km/24 h and did not differ significantly between C57Bl/6, cat^n (4.725 ± 0.15, $n=6$) and cat^{++} (5.11 ± 0.33, $n=8$). For comparison, treadmill training was performed using a previously established protocol [16]. After i.p. injection of heparin (Liquemin, Roche Pharma AG, Switzerland) mice were euthanized by CO_2 inhalation. Blood samples were collected from the heart for EPCs measurement. The aortas and soleus muscles were immediately frozen in liquid nitrogen and kept at –80°C for further analysis. Permission for this study was provided by the regional government and the experiments were performed according to the guidelines for the use of experimental animals as given by 'Deutsches Tierschutzgesetz' and to the 'Guide for the care and use of laboratory animals' of the US National Institutes of Health.

Fluorescence-activated cell sorter analysis (FACS)

Circulating EPCs in mouse peripheral blood were counted using FACS Calibur (Beckton Dickinson, USA). Aliquots of 100 µl of mouse peripheral blood were incubated with an antibody combination of anti CD3-APC, anti Flk-1-PE and anti CD34-FITC or anti CD3-APC, anti Flk1-PE and anti Sca-1-PE or anti CD3-APC, anti Flk-1-PE (all BD Pharmingen, USA) and anti CD133-FITC (eBioscience, Natutec, Germany). After an incubation period of 15 min at room temperature the erythrocytes were lysed using FACS lysing solution (BD Biosciences, San Jose, CA) and after several washing steps the remaining cells were fixed for FACS analysis. Isotype specific antibodies served as controls in every experiment (BD Pharmingen, eBioscience). To quantify the amount of EPCs in the mouse blood, only cells negative for the marker CD3 and double positive for CD34 and Flk-1 ($CD34^+/Flk\text{-}1^+$) or negative for CD3 and double positive for Sca-1 and Flk-1 ($Sca\text{-}1^+/Flk\text{-}1^+$) or negative for CD3 and double positive for CD133 and Flk-1 ($CD133^+/Flk\text{-}1^+$) were counted as EPCs. Another set of experiments was done for confirmation of our results with another FACS analyser (BD Coulter) using the same antibodies combinations except for CD3-APC which was replaced by CD3-PerCP (BD Bioscience, USA).

RT-PCR

Expression of transgene-specific mRNA in hearts of cat^{++}, cat^n and C57Bl/6 was analysed by RT-PCR using three different pairs specific to human catalase cDNA. The sense primers were TTCTGTTGAAGATGCGGCG (S1), TTTAACGCCATTGCCACA (S2), antisense-primers TGTTGTTCCGGAGCACCA (AS1), TCCGCACTTCTCCAGAAT (AS2), 1st primers pair S1/AS1, 2nd pair S2/AS1, 3rd pair S1/AS2.

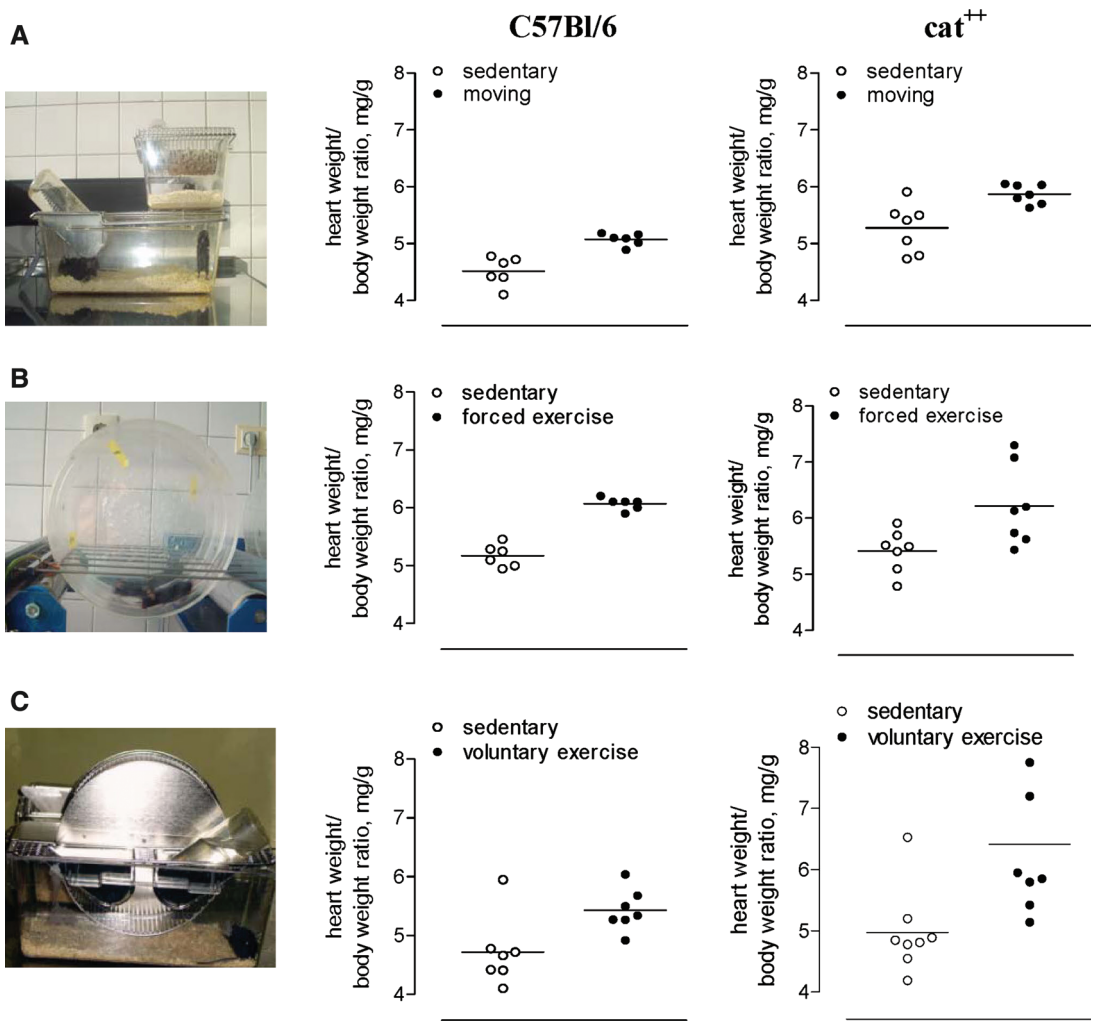

Figure 1. Details of the cages used to house and to exercise mice and heart weight/body weight ratio (mg/g) in sedentary and freely moving (A), forced exercised (B) and voluntary exercised (C) C57Bl/6 and cat^{++} mice. All exercise protocols significantly increased heart weight/body weight ratio in C57Bl/6 and cat^{++} mice almost identically when compared to sedentary singularized controls (n=6–7, p>0.05).

Western blotting

Mice thoracic aortas and bone marrow extracts were flash frozen in liquid nitrogen, homogenized, solubilized in lysis buffer [17] and centrifuged for 10 min at 100 x g. Supernatants were stored at –80°C until used for western blots. Western blot analysis was performed as described previously [16] using a monoclonal antibody directed against eNOS (Transduction Laboratories, Lexington, Kentucky) and a polyclonal antibody for catalase (Calbiochem, Darmstadt, Germany). Blots were developed using enhanced chemiluminescence (Roche, Mannheim, Germany) and exposed to x-ray film. The autoradiographs were analysed by densitometry (Geldoc, Bio-Rad, Muenchen, Germany). Total protein levels were determined by the Bradford method [18].

Measurement of blood pressure and heart rate

Systolic blood pressure (sBP) and heart rate were measured in awake male cat^{++} (n=8), cat^{n} (n=8) and C57Bl/6 (n=6) at 3–4 months of age using an automated tailcuff system (Visitech Systems, Apex, NC) as described previously [19]. In some experiments cat^{++} and cat^{n} mice (n=8, each) were treated with the catalase inhibitor aminotriazole (670 mg/kg/day, dissolved in drinking water) for 14 days. In another subset of experiments cat^{++} (n=6) and cat^{n} (n=5) and eNOS^{n} and eNOS^{++} (n=6 each) were treated for 28 days with N$^{\omega}$-nitro-L-arginine (L-NA, 100 mg/kg BW/day) and sBP and heart rate were recorded before, during and after the treatment period.

Substances and solutions

All chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany) in analytical grade.

Statistics

All data were analysed by standard computer programs (GraphPad Prism PC Software, Version 3.0,

Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (SEM). Significant differences were evaluated using either Newman-Keuls Multiple Comparison Test following OneWay-ANOVA or students *t*-test. A *p*-value below 0.05 was considered as significant.

Results

Characterization of transgenic mice

Briefly, cat^{++} showed human catalase mRNA expression as evidenced by transgene-specific PCR (Figure 2A) and increased catalase protein expression in both conductance (Figure 2B) and myocardial resistance vessels and associated with an increase of catalase mRNA, protein and activity [13]. Furthermore, there was a marked reduction of endothelial steady-state concentration of endogenous H_2O_2 measured by dichlorofluoresceine fluorescence in cat^{++} mice [12,13]. Since Tie-2 may also be expressed in neutrophils [20] we have measured catalase protein expression in the bone marrow extracts of cat^{n} and cat^{++} and have found no difference between the strains (Figure 2B). Furthermore, no difference between cat^{n} and cat^{++} was found by measurements of dihydroethidine fluorescence in leucocytes using FACS analysis suggesting absence of catalase over-expression in non-vascular cells [12]. Taken together, these data strongly suggest endothelial-specific over-expression of catalase and reduction of endogenous vascular H_2O_2 in our transgenic cat^{++} mouse model.

Reduction of blood pressure is one obvious phenotype of cat^{++} [12]. In the animals used for this study, we found a similar decrease of sBP from 116±2.6 mmHg (cat^{n}) to 101.4±2.5 mmHg in cat^{++} (n=8, p=0.0007). This effect was completely inhibited by treatment of mice with aminotriazole (117.4±4.0 mmHg, n=8, p=0.7653). The difference in sBP between cat^{n} and cat^{++} remained after inhibition of eNOS with oral L-NA treatment for 3 weeks (Figure 2C) suggesting that eNOS is not involved in sBP reduction in cat^{++}. Both findings confirm previous observations in these mice [12,13]. The $eNOS^{++}$ mice used in this study showed a 3.32±0.32-fold stronger aortic eNOS expression (n=7, p<0.001) as compared to $eNOS^{n}$. This was associated with a decrease of sBP from 118.1±2.4 mmHg in $eNOS^{n}$ to 105.6±3.3 mmHg in $eNOS^{++}$ (n=6, p=0.0151). In contrast to cat^{++}, this decrease of blood pressure was completely dependent on eNOS activity as evidenced by oral treatment with L-NA which increased sBP to a similar degree in $eNOS^{n}$ (134.5±3.1 mmHg, n=6) and $eNOS^{++}$ (132.9±2.1 mmHg, n=6, p=0.343).

The effect of training induced by different exercise protocols

To evaluate the efficacy of training we measured heart weight/body weight ratio. As shown in Figure 1, all training protocols significantly increased heart weight/body weight ratio in C57Bl/6 mice to a comparable extent suggesting a comparable cardiovascular adaptation to the different exercise protocols. Similar effects were observed in cat^{++} (Figure 1) demonstrating that the response to the each exercise protocol was not different between transgenic mice and C57Bl/6. Skeletal muscle citrate synthase activity was used as a metabolic marker for oxidative capacity to check for the changes in skeletal muscle energetics. While citrate synthase activity was increased in moving mice (55.5±4.3 mU/mg protein, n=5) and after forced physical activity (141.3±15.4 mU/mg protein, n=7) as compared to sedentary controls (40.3±2.8 mU/mg protein, n=7, p<0.05), we were unable to detect such a difference in voluntary running mice (55.2±5 mU/mg protein, n=7) vs sedentary controls (57.8±1.4 mU/mg protein, n=8, p=0.6038). However, voluntary training significantly increased soleus weight relative to body weight (0.356±0.03 vs 0.280±0.005 mg/g, n=4–5, p=0.027) and relative to tibia length (5.40±0.34 vs 4.17±0.20 mg/cm, n=4–5, p=0.0126).

The effect of training on the number of EPCs in C57Bl/6 mice

In a first set of experiments we used the stem cell marker CD34 and the endothelial cell marker Flk-1 to detect the effect of different training protocols on circulating EPCs. As shown in Figure 3, there were no changes of circulating EPCs induced by exercise. Likewise, no alterations in circulating CD34/Flk-1 double positive cells were detected after forced exercise (101.6±22.2 vs 90.4±17.1, n=8 each, p>0.05) and after voluntary running (81.8±9.9 vs 68.9±8.0, n=8 each, p>0.05) when another FACS analyser (BD Coulter) and the same antibodies combinations except for CD3-APC which was replaced by CD3-PerCP were used to count for EPCs.

To scrutinize this result, we investigated the effects of the same exercise protocol (voluntary running) on EPCs by using the three different stem cell markers CD34, Sca-1 and CD133 in combination with Flk-1. Again, there were no changes in EPC counts (Figure 4). Similarly, measurements using another FACS analyser (FACS Calibur) confirmed no changes in EPC numbers (369±109 vs 425±147 Sca1/Flk-1 double positive cells, n=5–6, p>0.05 or CD34/Flk-1 double positive cells (Figure 3C)). Likewise, we observed that the number of EPCs identified with Sca-1/Flk-1 was unchanged in freely moving compared to sedentary mice (577±128 vs 425±147 per 500 000 events, n=5, p=0.4588). The same was true when we used CD133/Flk-1 to identify EPCs in mice which underwent forced physical activity compared to sedentary controls (39±11 vs 36±17 per 500 000 events, n=6, p=0.8988).

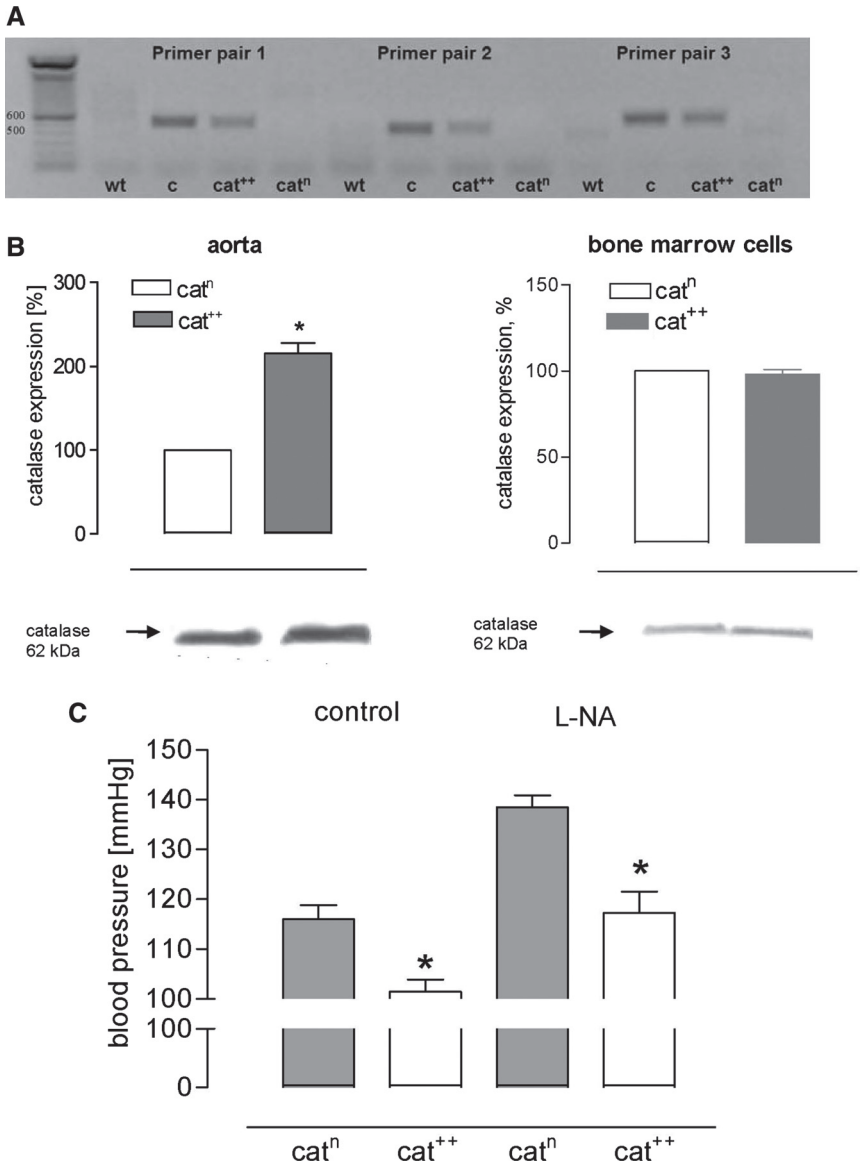

Figure 2. Evidence for functionally active over-expression of human catalase in transgenic mice. (A) Transcription of human catalase in heart of cat^{++} as evident by transgene-specific RT-PCR. The expected size bands of 510, 449 and 557 bp were detected in transgene-positive mice (cat^{++}) using three different pairs of primers specific for human catalase cDNA, while there were no signals in transgene-negative littermates (cat^{n}). Total mRNA from heart of C57Bl/6 mice served as negative control (wt), mRNA isolated from human umbilical vein served as positive control (c). (B) Catalase protein expression measured by western blotting in the aortic homogenates and in the bone marrow cells of cat^{++} and cat^{n}. (C) Effect of NO-synthase inhibitor L-NA (100 mg/kg BW/day) on systolic blood pressure in cat^{++} and cat^{n}. Measurements were obtained in resting awake animals using a tail-cuff method (* n=5-6, $p<0.001$, one way ANOVA).

The effect of eNOS and catalase on the number of EPCs in transgenic mice

All experiments in transgenic mice were done using CD34/Flk-1 to identify EPCs. To investigate an effect of H_2O_2 on the number of circulating EPCs we compared cat^{++}-mice with cat^{n}-mice but found no effect of the transgene (Figure 5A). In contrast, treatment of these mice with aminotriazole resulted in a large decrease of circulating EPCs suggesting that increased H_2O_2 is the underlying mediator of this effect (Figure 5A). It has been published that cat^{++} have hypotension and that inhibition of catalase in these mice by aminotriazole increases sBP back to normal values [12]. Thus, blood pressure might be a confounding factor in our study. To further investigate a possible role of blood pressure we used two other strains of transgenic mice, $eNOS^{-/-}$ having hypertension [19] and $eNOS^{++}$ having hypotension [12]. As shown in Figures 5B and C the numbers of EPCs were unchanged in these animal models suggesting

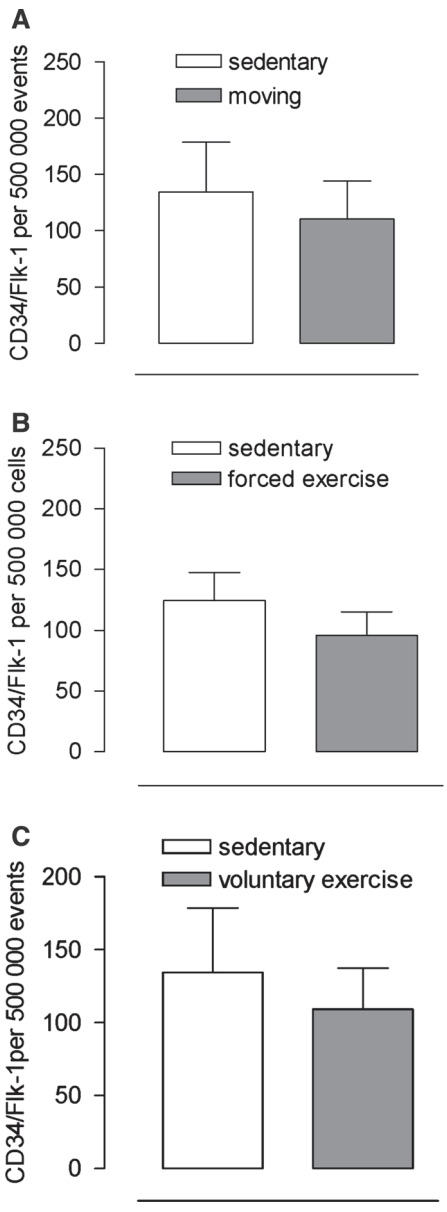

Figure 3. No difference in circulating EPCs measured as CD34/Flk-1 positive cells using FACS Calibur (Beckton Dickinson) in peripheral blood of freely moving (A), forced (B) and voluntary (C) exercised C57Bl/6 mice (n=5–9, p>0.05).

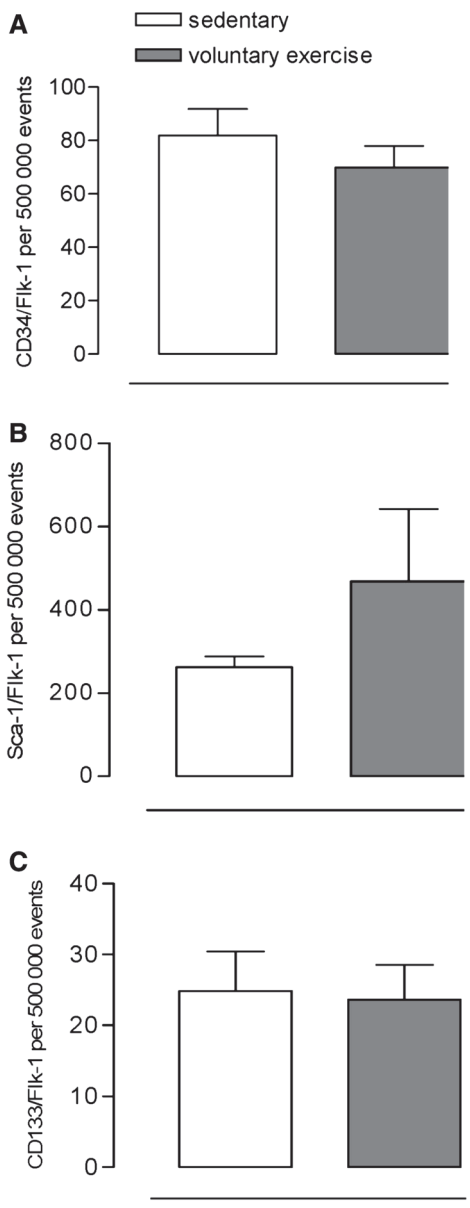

Figure 4. Voluntary exercise training had no effect on circulating EPCs in C57Bl/6 mice as measured by FACS analyser (BD Coulter) using different stem cell markers. Circulating EPCs were defined (A) as CD34/Flk-1 (n=8, p=0.365); (B) as Sca-1/Flk-1 (n=7–8, p=0.292) and (C) as CD133/Flk-1 (n=6–7, p=0.876) double positive cells.

that neither eNOS activity nor blood pressure appears to be important for the number of circulating EPCs in resting healthy mice.

The effect of the catalase transgene on circulating EPCs after exercise training

A comparison of the response of cat^{++} to two different training protocols showed a strong and significant increase of circulating EPCs induced by forced physical activity (Figure 6A) and voluntary exercise (Figure 6B). These data suggest that H_2O_2 inhibits exercise-induced augmentation of circulating EPCs in cat^{n} and C57Bl/6 mice (Figure 3). In accordance with this suggestion we found that aminotriazole completely inhibited the augmenting effect of exercise on circulating EPCs in cat^{++} (Figure 6C).

Discussion

The aim of this study was to investigate the effects of vascular H_2O_2 on the number of circulating EPCs and on changes thereof in response to a 3–4 weeks moderate exercise training period. Our major new finding is that H_2O_2 as an important component of total vascular oxidative stress inhibits exercise-induced

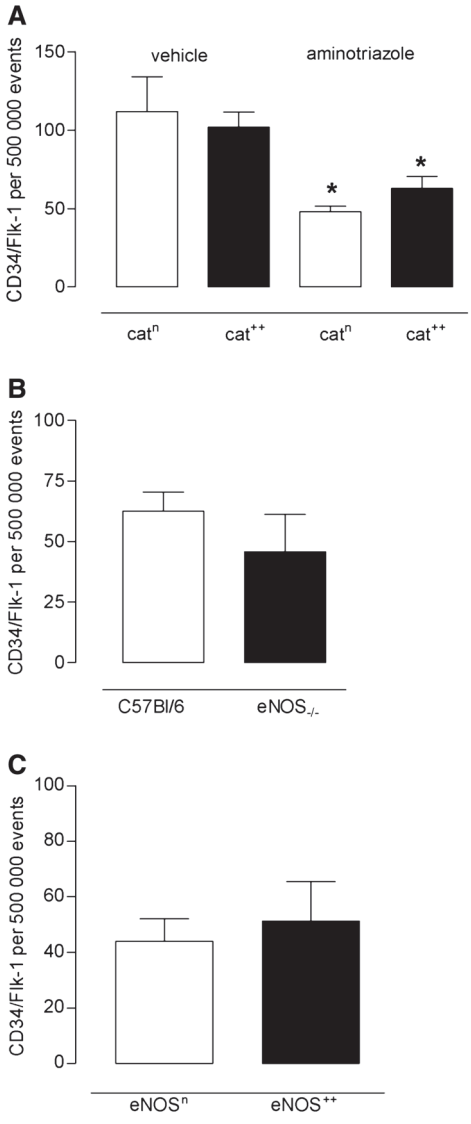

Figure 5. Circulating EPCs in peripheral blood of different transgenic mouse strains. (A) There was no difference between basal EPC levels in cat^n and cat^{++} mice. Oral treatment with catalase inhibitor aminotriazole significantly decreased EPCs in peripheral blood of cat^n and cat^{++} (* n=5–8, p<0.05). (B) Circulating EPCs in eNOS knockout mice ($eNOS^{-/-}$) and (C) mice with endothelium-specific over-expression of eNOS ($eNOS^{++}$). (C) Neither eNOS knockout (n=6, p=0.6625) nor insertion of eNOS gene (n=7, p=0.358) changed EPC counts in resting mice.

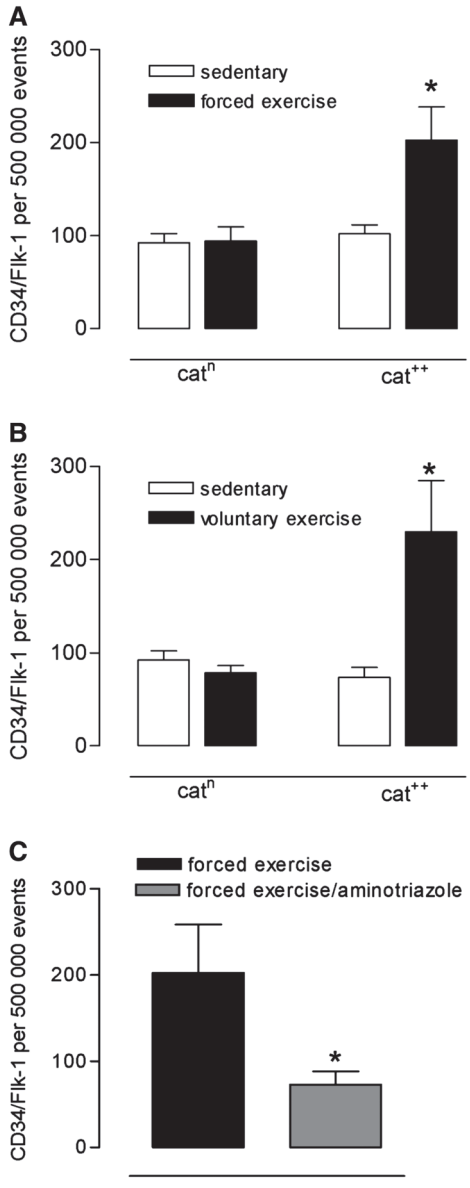

Figure 6. Moderate forced (A) and voluntary (B) exercise training strongly increased circulating EPCs in cat^{++} mice and this was completely abolished by treatment with catalase inhibitor aminotriazole (C) (n=5–8, p<0.05).

upregulation of circulating hematopoietic stem cells with endothelial progenitor capacity.

Vascular H_2O_2 is known to be an important part of vascular oxidative stress and exerts several deleterious effects in the cardiovascular system such as increased smooth muscle proliferation, stimulation of inflammation, promotion of the atherosclerotic process, generation of tissue-toxic hydroxyl radicals and alterations of vascular tone [21]. Here we describe a new and potentially hazardous activity of vascular H_2O_2, i.e. a reduction of the number of circulating EPCs. Several lines of evidence support this suggestion. Aminotriazole, which inhibits catalase activity and accumulates tissue H_2O_2, strongly decreased circulating EPCs in resting animals. Likewise, endothelial-specific over-expression of catalase and subsequent reduction of vascular H_2O_2 [12,13] resulted in an increased number of circulating EPCs in response to different exercise protocols. Finally, the exercise-induced increase of EPCs was completely abolished in cat^{++} treated with aminotriazole. These data suggest that H_2O_2 is involved in the physiologic non-ischemic regulation of the number of circulating EPCs both at rest and by exercise.

According to previous investigations, the number of EPCs was increased by non-ischemic exercise in mice [10] but not in patients [22,23]. To further investigate the

role of non-ischemic exercise on the number of circulating EPCs, we used several different exercise protocols. The different protocols appear to be equally effective as indicated by a comparable increase of heart weight/body weight ratio. However, the distance voluntarily running mice ran each night was almost 10 times greater than the daily distance made by mice subjected to forced physical activity. Furthermore, freely moving mice and those who underwent daily forced physical activity appear to be much more exposed to stress factors such as the aggressive nature of the C57Bl/6 strain in freely moving mice living in groups and the experimental procedure of forced running mice in rotating wheels. While such maximal strains are absent in singularized voluntarily running mice, this type of exercise training rather matches endurance training with a lower but more constant training intensity. In addition, voluntary running matches the preferred running time and speed for each individual mouse. In general, mice appear to voluntarily run in short bouts of 2–3 min at a mean speed of 2–3 km/h for a total of 100–120 bouts per night [24]. While individual bouts may be of high intensity, they are of short duration and do not invoke comparable adaptation in mitochondrial enzyme activity in slow-twitch soleus muscle. The absence of increase in citrate synthase activity has been reported previously [25,26]. Although we didn't analyse voluntary running patterns in detail, many aspects such as total running distance, changes of heart weight/body weight, soleus weight/ body weight and soleus weight/tibia length ratios and daily running distance demonstrate that mice run sufficiently in our voluntary model of exercise.

Identification of EPCs by FACS analysis relies on the combined expression of proteins indicative for stem cells such as CD34, CD133 and Sca-1 and for endothelial cells such as the VEGFR2 (Flk-1). Cell culture experiments have shown that human EPCs are not a homogenous population of stem cells. For example, early and late outgrowth EPCs can be differentiated [27]. Furthermore, Sca-1 positive non-bone marrow-derived progenitor cells are able to differentiate into both, endothelial and smooth muscle cells [28]. It was also noted that Sca-1 is expressed on mature endothelial cells and this might hold true for CD34 as well [5]. Accordingly, CD133/Flk-1 double staining is currently considered to be the most reliable identification for EPCs [29,30]. However, in mice the number of these cells is extremely low and this makes a quantitative evaluation very difficult. Therefore, we have compared the data obtained with all three stem cell markers and found similar results. The only trend for an increase of circulating EPCs we have detected was when Sca-1/Flk-1 double positive cells were counted following voluntary running and a similar observation has been made previously [10]. Thus, it appears indeed important not to rely on measurements with just one stem cell marker [30].

Blood pressure might [31] or might not [7] impact on the number of circulating EPCs in humans. In mice, we observed an exercise-induced increase of EPCs in cat^{++} only, a strain showing both decreased steady state levels of vascular H_2O_2 and reduced blood pressure as compared to cat^{n} and C57Bl/6. Thus, blood pressure might have been a confounding factor in our study. However, resting cat^{++} and cat^{n} treated with aminotriazole showed a comparable decrease of EPCs, although aminotriazole treatment increased blood pressure in cat^{++} but not in cat^{n}. In order to further clarify whether blood pressure changed the effect of exercise on the number of EPCs in our study, we evaluated other transgenic mouse strains. We generated mice using the same promotor and enhancer element as in cat^{++} but inserted bovine eNOS ($eNOS^{++}$). Furthermore, eNOS knock-out mice ($eNOS^{-/-}$) were investigated.

While $eNOS^{-/-}$ have hypertension, $eNOS^{++}$ have hypotension which was completely dependent on treatment with the NOS-inhibitor L-NA, suggesting NO-dependent reduction of blood pressure. All of these different animal strains didn't differ in terms of basal (resting) number of circulating EPCs. These data strongly suggest that changes of blood pressure unlikely change the number of circulating EPCs in mice. A similar situation appears to hold true for the effects of exercise. Aminotriazole completely inhibited exercise-induced up-regulation of circulating EPCs in cat^{++}-mice and increased blood pressure at the same time. However, treatment of cat^{n} with animotriazole didn't change blood pressure and EPC-response to exercise, suggesting that changes of blood pressure had no effect on exercise-induced increase of circulating EPCs in our strains of mice.

Maintenance and mobilization of EPCs in the bone marrow is determined by the local microenvironment, which consists of fibroblasts and endothelial cells. Recently we have found significantly increased level of vascular MMP-9, a key matrix metalloproteinase involved in mobilization of EPCs in cat^{++} mice (our unpublished data). H_2O_2 is able to accumulate extracellularly in the tissue and survive long enough to induce paracrine functions, even in more distant cells. We speculate that vascular H_2O_2 can diffuse from the vascular niche to the stem cell niche of the bone marrow and have an inhibitory effect on the local MMP-9. This effect is abolished in mice with endothelial-specific over-expression of catalase. Further studies are necessary to clarify molecular mechanisms of H_2O_2-dependent bone marrow MMP-9 regulation.

In summary, we reported a new and potentially hazardous activity of vascular endogenous H_2O_2, i.e. a reduction of the number of circulating EPCs. These data suggest that H_2O_2, a known component of vascular oxidative stress, likely contributes to the impairment of important stem cell-induced vascular repair mechanisms in cardiovascular disease.

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