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The role of nitric oxide in remodeling of capillary network in rat interscapular brown adipose tissue after long-term cold acclimation

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Summary. Cold exposure has been shown to increase blood flow in interscapular brown adipose tissue (IBAT). The aim of the present study was to evaluate the role of the L-arginine-nitric oxide ($^{\bullet}NO$) pathway on IBAT capillary network remodeling and its possible correlation with superoxide anion radical ($O_2^{\bullet-}$). In the rats that received L-arginine (2.25%) or N^G-nitro-L-arginine methyl ester (L-NAME, 0.01%) as a drinking liquid and maintained at room (22±1°C) or low (4±1°C) temperature for 45 days, IBAT capillaries were analyzed by stereology and observed by light and electron microscopy. Additionally, endothelial $^{\bullet}NO$ synthase (eNOS) expression, nitrotyrosine immunoreactivity and both copper zinc superoxide dismutase (CuZnSOD) enzyme activity and immunohistochemical localization were examined. Stereological analyses of IBAT show that the capillary volume density, as well as capillary-to-brown adipocytes ratio, are increased in cold. L-arginine treatment increases, while L-NAME decreases both parameters, compared to respective controls. Those changes were accompanied by capillary dilatation observed by light and electron microscopy. The activity of CuZnSOD is lower in control cold-acclimated rats, as well as in both L-arginine-treated groups, when compared to control animals acclimated to room temperature. L-NAME treatment attenuates the effects both of cold and L-arginine on CuZnSOD and increases immunopositivity for CuZnSOD in room temperature-acclimated rats. Our results show that $^{\bullet}NO$ induces remodeling of the IBAT capillary network by angiogenesis, and presumably that interaction with $O_2^{\bullet-}$ has a role in that modulation. The increased eNOS expression accompanied by an increased nitrotyrosine immunoreaction observed in both L-arginine-treated groups compared to corresponding controls strengthens this hypothesis.

Key words: Capillary network, Interscapular brown adipose tissue, Nitric oxide, CuZnSOD, Cold

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Introduction

Interscapular brown adipose tissue (IBAT) is a unique tissue, essential for producing heat and maintaining body temperature in mammals (Kuroshima, 1993). Chronic exposure to cold is accompanied by an increase in tissue mass, which enhances its metabolic and thermogenic activity (Smith and Roberts, 1964; Petrovic et al., 2006). It is well known that cold-induced IBAT thermogenesis is regulated by the sympathetic nervous system via noradrenergic innervation (Ma and Foster, 1984). The IBAT is very rich in capillaries (Foster and Frydman, 1978), and their extreme enlargement is considered to depend on the increase in both the number of brown adipocytes and capillary density. Moreover, it was found that anti-angiogenic agents can prevent the tissue growth (Nagashima et al., 1994).

The regulation of blood flow in IBAT has been extensively studied. Numerous studies show that this process is redox sensitive and suggest the role of nitric oxide ($^{\bullet}NO$) in its regulation (Foster and Frydman, 1978; Ma and Foster, 1984; Fukumura et al., 2001; Kikuchi-Utsumi et al., 2002). It was found that treatment with N^G-nitro-L-arginine methyl ester (L-NAME) prevents cold-induced increase of blood flow in rat IBAT (Nagashima et al., 1994). Furthermore, L-NAME, *in vivo* and *in vitro*, reduces the local IBAT temperature, suppresses the thermogenic response, and inhibits the cold-induced tissue hyperplasia (Saha et al., 1996, 1997). We previously also reported that L-NAME

administration reduces the cold-induced IBAT growth which suggested that the total tissue homeostasis depends on the metabolic, structural and redox homeostasis (Petrovic et al., 2005).

It has also been shown that •NO in IBAT is produced by the activity of inducible and endothelial nitric oxide synthase (iNOS and eNOS) and that the absolute amount of •NO in IBAT and its physiological response depends on the expression of individual NOS isoforms, as well as on the functional state of the tissue (Kikuchi-Utsumi et al., 2002; Petrovic et al., 2005). Also, the functional response of •NO in IBAT and other tissues is modulated by the degree of interaction with superoxide anion radical ($O_2^{\bullet-}$), i.e. the activity of superoxide dismutase (SOD) (Czapski and Goldstein, 1995; Petrovic et al., 2005). This suggests that the effect of •NO on the angiogenic potential of IBAT is complex too, and includes a wider redox homeostasis in the tissue, primarily the role of copper zinc SOD (CuZnSOD), the most abundant isoforms in the majority of cell compartments.

For these reasons, our objective in the present paper was to investigate the effect of •NO on the capillary network in IBAT of room temperature and long-term cold-acclimated rats, modulating its production by a chronic (45 days) treatment with L-arginine or L-NAME. The remodeling of the capillary network in IBAT was studied by light and electron microscopy, stereological analysis of the capillary volume density, accompanied by eNOS expression, CuZnSOD and nitrotyrosine immunohistochemical localization and monitoring of CuZnSOD enzyme activity, which is presumed to be able to affect the functional response of •NO in IBAT.

Materials and methods

Animals and experimental design

Male Mill Hill hybrid hooded (*Rattus norvegicus*, Berkenhout, 1769), 4-month-old rats were divided into three main groups. The first group received L-arginine·HCl (2.25%), a substrate for NOSs, and the second group received L-NAME·HCl, (0.01%), an inhibitor of NOSs in drinking water, for 45 days. The doses of L-arginine and L-NAME used here did not have toxic effects on the animals, as confirmed elsewhere (Saha et al., 1996; Petrovic et al., 2005). The third group served as a control. Animals of all three groups were additionally divided into two subgroups: one was kept at 22±1°C, and the other in the cold room at 4±1°C. The animals were kept in individual cages, with food and water ad libitum. Each experimental group consisted of six animals. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals.

The animals were sacrificed by decapitation, the IBAT dissected out within 3 min after death, and thoroughly rinsed with physiological saline to remove traces of blood. The tissue was homogenized (a Janke and Kunkel Ka-Werke Ultra-Turrax homogenizer, 0-4°C) in 0.25 mol/l sucrose, 0.1 mmol/l EDTA, and 50 mmol/l Tris·HCl buffer, pH 7.4. The homogenates were sonicated as suggested by Takada et al. (1982).

Activity of superoxide dismutase

Superoxide dismutase activity was determined by the method of Misra and Fridovich (1972). The activity was expressed in U mg^{-1} protein. The total specific SOD activity and manganese SOD (MnSOD) activity after inhibition with a 4 mmol/l KCN were measured, and then CuZnSOD activity calculated. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autooxidation of adrenaline under the appropriate reaction conditions.

Immunohistochemistry

Immediately after removal, the samples of IBAT were fixed in a 10% formaldehyde solution at 4°C overnight and processed routinely for embedding in paraffin. A series of 5 μm thick IBAT sections were deparaffinized and rehydrated. Immunoreactivity was assessed by the avidin-biotin-peroxidase method (Santa Cruz Biotechnology manual). The sections were incubated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature to block endogenous peroxidase, followed by three washes in 0.015 mol/l phosphate-buffered saline (PBS; pH 7.4) of 5 minutes each, and incubation with a 1.5% normal goat serum (ABC Staining System, Santa Cruz Biotechnology) in PBS for 60 minutes at room temperature to block non-specific binding. The primary antibodies against CuZnSOD (Santa Cruz Biotechnology) and nitrotyrosine (Abcam) were polyclonal antibodies produced in rabbit. The sections were incubated with the primary antibody in PBS (1:200 dil.) overnight at 4°C, followed by two 5 minutes PBS washes, then incubated with 1:200 IgG biotinylated serum goat anti-rabbit (ABC Staining System, Santa Cruz Biotechnology) in PBS for 30 minutes at room temperature, followed by two PBS washes of 5 minutes each. AB reagent (ABC Staining System, Santa Cruz Biotechnology) was added for 60 minutes at room temperature, followed by three PBS washes of 5 minutes each, and then incubated with 0.02% H_2O_2 and 0.075% diaminobenzidine (Sigma) in 0.05 mol/l Tris buffer, pH 7.6, for 10 minutes in a dark room. The sections were rinsed in distilled water, counterstained with hematoxylin, and then observed with a Leica DMLB microscope.

SDS-PAGE and Western blotting

Proteins were resolved according to Laemmli (1970). Ten mg protein aliquots were boiled and electrophoresed in 7.5% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane and

incubated with rabbit polyclonal antibody against eNOS (Chemicon International) at 1:200 dilution in TBS-T supplemented with 5% BSA, overnight in a cold room. After multiple washes in TBS-T, the membrane was incubated with horseradish peroxidase-conjugated IgG secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilution. For specific band detection, the membrane was covered by luminol-based chemiluminescent substrate for 3 minutes. Immediately after, a piece of x-ray Hyperfilm MP (Amersham API) was placed over the blot and exposed 1 min. The film was developed, scanned and a quantitative analysis of immunoreactive bands was done by an ImageQuant software. Volume is the sum of all the pixel intensities within a band; 1 pixel = 0.007744 mm^2.

Electron-microscopic examination

Immediately after removal, the samples of IBAT were cut into small pieces, fixed in 2.5% glutaraldehyde in a 0.1 mol/l phosphate buffer (pH 7.2), and postfixed in 2% osmium tetroxide in the same buffer. The specimens were dehydrated through a series of alcohol solutions of increasing concentration and embedded in Araldite. The blocks were trimmed and cut with glass knives on an LKB III ultramicrotome. The thin sections were contrasted with uranyl acetate and lead citrate, and observed with a Philips CM 12 electron microscope.

Stereological analysis. Counts for stereological analysis were done on 1 μm thick plastic sections of IBAT. Transverse sections were cut in series (three) or in steps (every 10th) from one to four blocks of IBAT, mounted on glass slides and stained with toluidine blue. The sections were then viewed by a microscope with a 100X lens. The volume density of capillaries was estimated using a derivation of Deless equation V/V=Pc/Pt, were Pc/Pt is the point fraction or the total points hitting the capillaries divided by the total points of a Weibel multipurpose test system (Aherne and Dunnhill, 1982). The same sections were further used for counting the number of capillaries per brown adipocyte and determination of the capillary-to-brown adipocyte ratio.

Protein content was estimated by the method of Lowry et al. (1951).

Analysis of variance (ANOVA) was used for within-group comparison of data obtained during experimental conditions. If the *F* test showed an overall difference, Student's t-test was used to identify significant differences ($p<0.05$).

Results

The capillary volume density results are given in Fig. 1. Long-term acclimation to cold increases the capillary volume density in IBAT ($p<0.005$). In both groups of animals treated with L-arginine the values are higher, and lower in L-NAME-treated animals, when compared to the corresponding controls kept at the same temperatures ($p<0.005$).

A histogram (Fig. 2) represents the number of capillaries per adipocytes, i.e. capillary-to-brown adipocyte ratio. The results show that L-arginine increases the ratio, and L-NAME decreases it.

The results of light and electron microscopy are presented in Fig. 3. In all cold-acclimated groups changes in the appearance of brown adipocytes are observable - the numbers of multilocular adipocytes are increased (Fig. 3B,D,F, left). Capillary dilatation is observed in the control cold-acclimated group (Fig. 3B, left), as well as in both L-arginine treated groups (Fig. 3C,D, left), at the level of the capillary network. In addition, the high rate of capillarization in these groups is accompanied by a higher blood flow, judging from the erythrocyte count in the capillary lumen. In the L-NAME treated group acclimated to room temperature (Fig. 3E, left), the capillaries are narrowed and the tissue appears avascular.

When observed under an electron microscope, IBAT capillaries showed more clearly the changes observed with light microscopy. Compared to room temperature (Fig. 3A, right), cold induces vasodilatation and increases the number of capillaries (Fig. 3B, right). Strong vasodilatation of capillaries is observed in L-arginine-treated animals (Fig. 3C, right), especially in cold-acclimated rats (Fig. 3D, right). L-NAME administration in room temperature-acclimated rats suppresses blood flow and, consequently induces capillary lumen narrowing and in some cases closes (Fig. 3E, right). Cold acclimation attenuates L-NAME effects on the capillary network (Fig. 3F, right), but

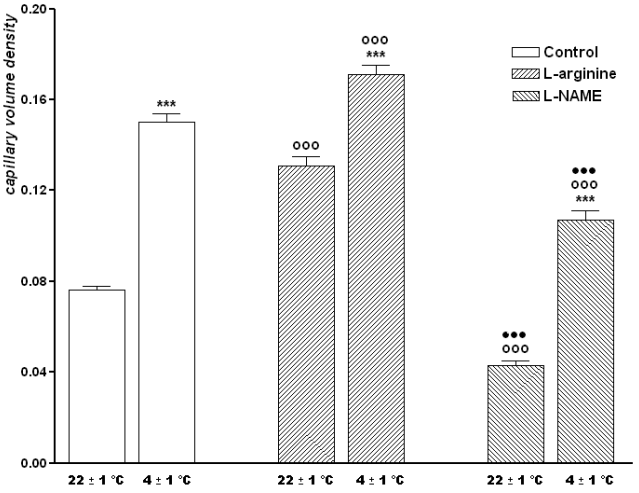

Fig. 1. The capillary volume density in IBAT. The values are the means ± S.E.M. *Comparison of the same treatments at different temperatures, ***$p<0.005$; °comparison of different treatments with the control maintained at the same temperature, °°°$p<0.005$; *comparison of L-NAME treatments with the L-arginine groups at the same temperature, ***$p<0.005$. Capillary volume density units mm^3/mm^3.

capillary density remains less than that observed in corresponding control.

Changes of the CuZnSOD activity in IBAT are presented in Fig. 4. A statistically significant lower enzyme activity is detected in cold-acclimated rats ($p<0.005$) and in both groups of animals treated with L-arginine ($p<0.05$), compared to the control group at room temperature.

Immunohistochemically, CuZnSOD (Fig. 5) is localized in the cytoplasm of brown adipocytes, except in the room temperature acclimated group of animals treated with L-NAME, where pronounced immunolocalization in brown adipocytes nuclei is observed.

Nitrotyrosine immunoreactivity was shown in Fig. 6. Among groups acclimated to room temperature, nitrotyrosine immunoreaction was stronger in both L-arginine and L-NAME-treated groups compared to the control group. Among groups kept at cold, nitrotyrosine immunopositivity was detected only in the L-arginine-treated group.

The eNOS immunoblot (Fig. 7A) showed the strongest eNOS band, among all groups, in the L-arginine-treated-group acclimated to room temperature. Quantification of eNOS bands by ImageQuant software

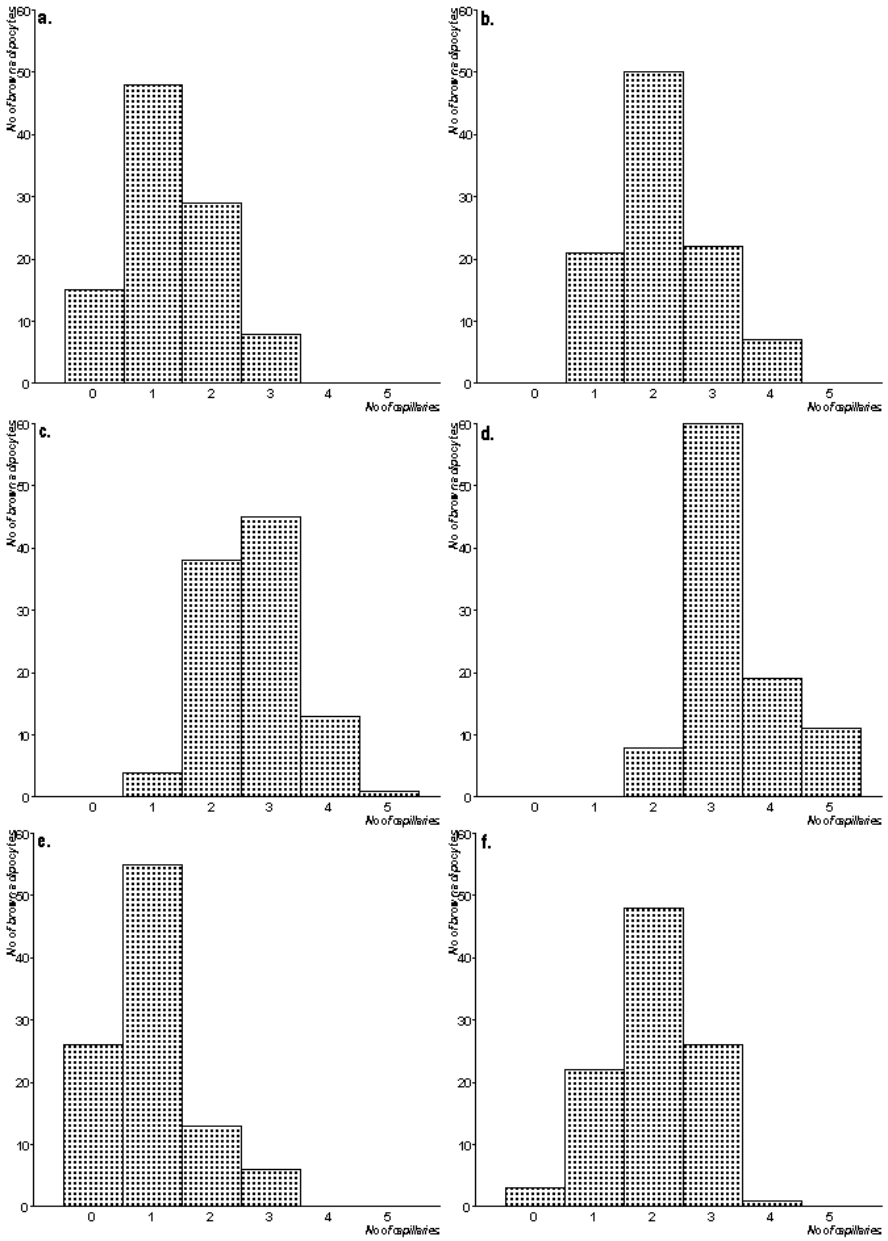

Fig. 2. A histogram represents capillary-to-brown adipocyte ratio. **A.** Control 22±1°C. **B.** control 4±1°C. **C.** L-arginine 22±1°C. **D.** L-arginine 4±1°C. **E.** L-NAME 22±1°C. **F.** L-NAME 4±1°C.

(Fig. 7B) revealed that in IBAT of rats kept at room temperature, the eNOS level was significantly higher in L-arginine-treated rats, while in L-NAME-treated rats it was decreased compared to the corresponding control ($p<0.005$ and $p<0.05$, respectively). Similarly, the eNOS protein level of cold acclimated rats in the L-arginine-treated group was significantly higher ($p<0.005$), while the level of this protein in L-NAME-treated rats was decreased, compared to the cold-maintained control ($p<0.005$). Besides, eNOS level was significantly decreased ($p<0.005$) in all cold-acclimated groups in comparison to appropriate groups maintained at room

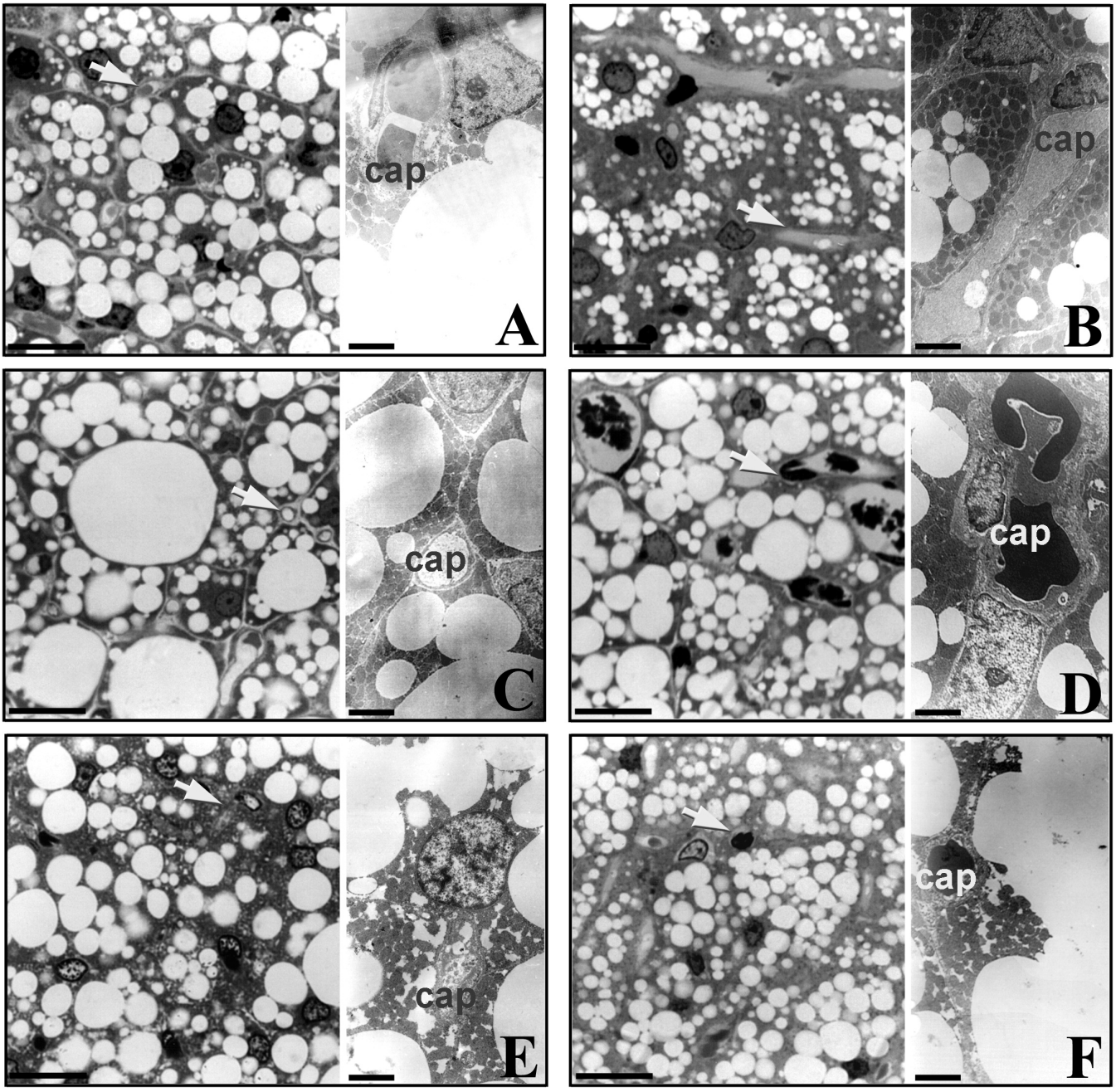

Fig. 3. IBAT structure (light microscopy – left in box) and capillary ultrastructure (electron microscopy – right in box). **A.** Control 22±1°C. **B.** control 4±1°C. **C.** L-arginine 22±1°C. **D.** L-arginine 4±1°C. **E.** L-NAME 22±1°C. **F.** L-NAME 4±1°C. White arrows: capillaries; cap: capillaries. Scale bars: light microscopy, 30 μm; electron microscopy, 5 μm.

temperature.

Discussion

The research carried out so far shows that acclimation of rats to low temperature induces IBAT hyperplasia, tissue mass increase, and enhances tissue blood flow (Smith and Roberts, 1964; Foster and Frydman, 1978; Saha et al., 1996, 1997; Petrovic et al., 2006). Stereological analysis presented in this paper shows that the blood circulation increase reflects the increase of capillary volume density and capillary count per brown adipocyte. Also, $^{\bullet}NO$ modulates chemodynamics in IBAT: L-arginine treatment increases both parameters, and L-NAME reduces them, either after a long-term cold or room temperature acclimation. Furthermore, the decrease in CuZnSOD activity in both L-arginine-treated groups accompanied with increased eNOS expression and increased nitrotyrosine immunopositivity in this experimental model indicates a possible interaction between $^{\bullet}NO$ and $O_2^{\bullet-}$ in modulation of angiogenesis, and a subsequent blood flow in IBAT. Possible mechanisms of these changes are discussed.

Blood flow in IBAT depends on the thermogenic state, is angiogenically dependent, and many autocrine, paracrine, and endocrine factors are included in its regulation. It is shown that $^{\bullet}NO$, endogenously produced in brown adipocytes, can directly modulate not only thermogenic IBAT activity, but also blood flow (Foster and Frydman, 1978; Ma and Foster, 1984; Nagashima et al., 1994; Fukumura et al., 2001; Kikuchi-Utsumi et al., 2002). In a similar experimental model, Saha et al.

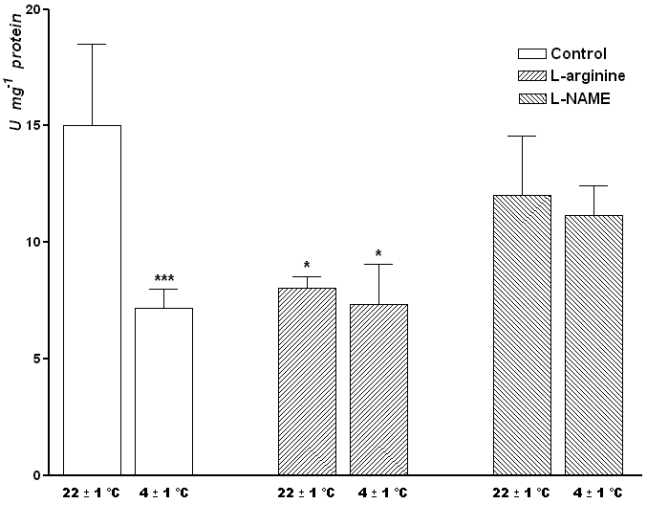

Fig. 4. CuZnSOD activity in the IBAT. Enzyme activity is expressed in U mg^{-1} protein. The values are the means ± S.E.M. (n=6). Comparison with the control maintained at room temperature, *p<0.05; ***p<0.005.

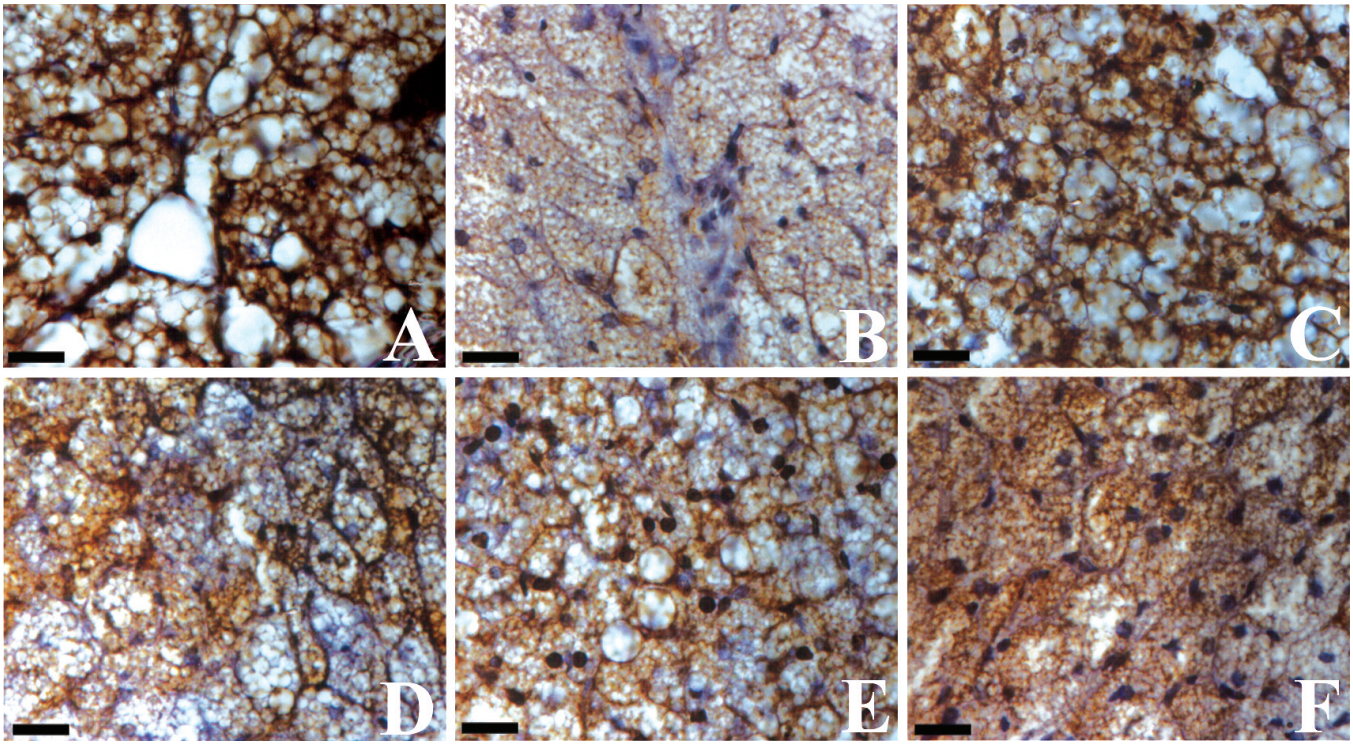

Fig. 5. Immunohistochemical localization of CuZnSOD in IBAT. **A.** Control 22±1°C. **B.** control 4±1°C. **C.** L-arginine 22±1°C. **D.** L-arginine 4±1°C. **E.** L-NAME 22±1°C. **F.** L-NAME 4±1°C. Scale bars: 30 μm.

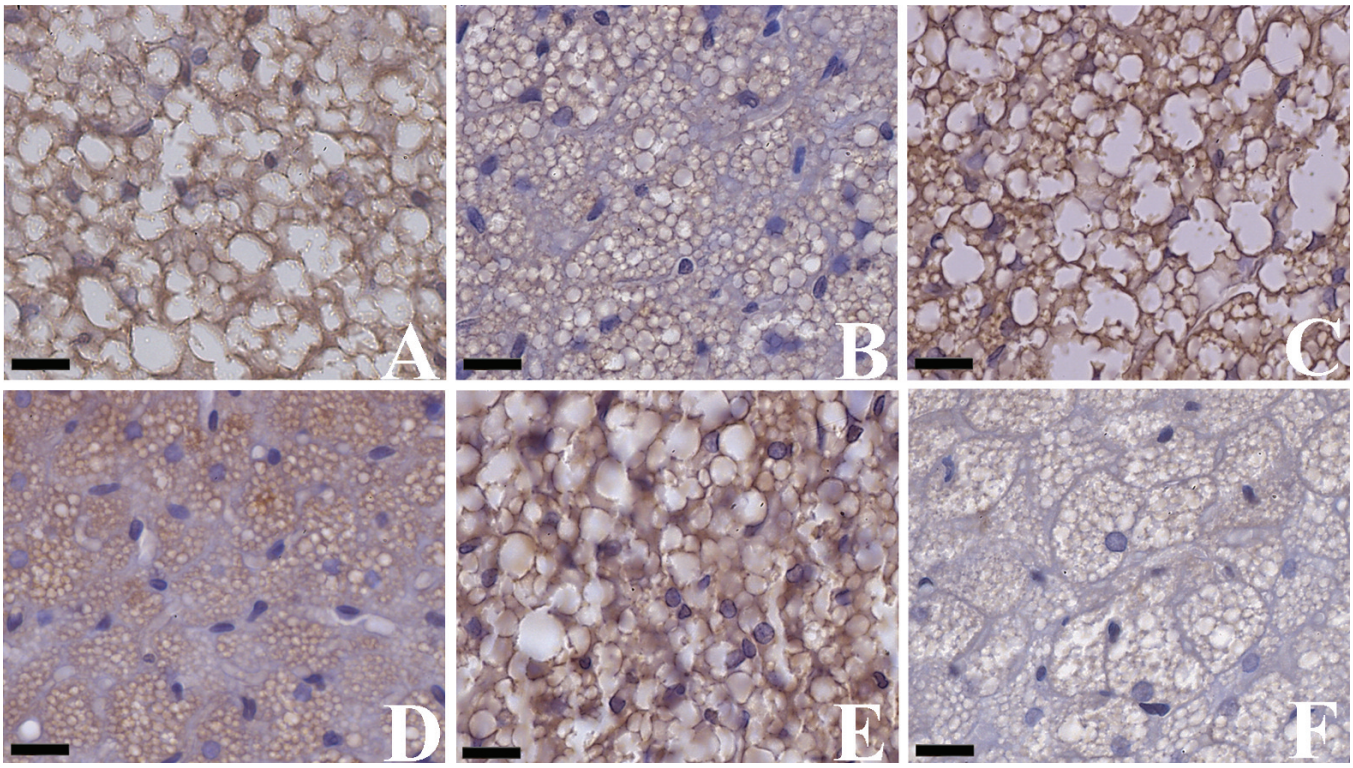

Fig. 6. Immunohistochemical localization of nitrotyrosine in IBAT. **A.** Control 22±1°C. **B.** control 4±1°C. **C.** L-arginine 22±1°C. **D.** L-arginine 4±1°C. **E.** L-NAME 22±1°C. **F.** L-NAME 4±1°C. Scale bars: 30 μm.

(1996) showed that L-NAME supplementing depresses oxygen consumption, DNA synthesis, cold induced hyperplasia, and noradrenaline stimulated •NO production in IBAT. Our earlier results show that long-term L-NAME treatment reduces the cold-induced mass increase of IBAT, whereas L-arginine treatment additionally increases the cold-stimulated expression of UCP1 (Petrovic et al., 2005). Detailed mechanisms through which •NO realizes this action in IBAT have still not been elucidated.

Kikuchi-Utsumi et al. (2002) have shown that sympathetic nerves, i.e. noradrenaline, can mediate vasodilatation through stimulating production of •NO, resulting in an increase in IBAT blood flow, which is completely abolished with L-NAME. The authors emphasized that IBAT is one of the mammal tissues with the highest eNOS expression. In agreement with this are the results of Nagashima et al. (1994), which show that noradrenaline, alpha, and beta agonists increase IBAT blood flow. This noradrenaline induced blood flow increase in IBAT is suppressed by L-NAME in a dose dependent way. Fredriksson et al. (2000) conclude that noradrenaline stimulates expression and secretion of the vascular endothelial growth factor (VEGF) in brown adipocytes' culture, while Asano et al. (1999, 2001) show that VEGF expression in IBAT is activated via adrenergic mechanisms during cold exposure. Inhibition

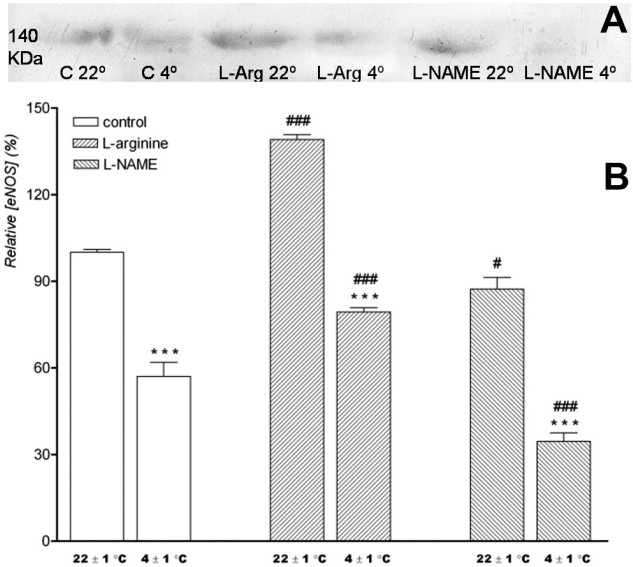

Fig. 7. The effects of L-Arg and L-NAME supplementation and different temperatures on eNOS protein level in IBAT **A.** Western blotting was performed using specific antibody against eNOS. The results of a representative experiment from three observations are shown. **B.** Data obtained after quantification of eNOS bands by ImageQuant software. Relative eNOS concentration is expressed in relation to the control acclimated to room temperature taken as 100%. The values represent the means ± SE from three independent experiments.

of *in vivo* •NO production results in inhibition of angiogenesis and vascular permeability induced by VEGF. The authors single out eNOS as the main isoform that mediates in VEGF induced angiogenesis (Fukumura et al., 2001; Kikuchi-Utsumi et al., 2002). Accordingly, the eNOS immunoblot from the present study showed among groups acclimated to room temperature the strongest eNOS expression in the L-arginine treated group. Nevertheless, long-term cold exposure generally decreased eNOS expression. However, L-arginine treatment increased, while on the other hand, L-NAME treatment decreased eNOS expression compared to the cold-acclimated control. Additionally, our previous study performed with the same experimental conditions revealed increased iNOS expression in both L-arginine-treated groups acclimated to room or low temperature compared to corresponding controls (Petrovic et al, 2005). These data strongly indicate that in IBAT •NO derived from both eNOS and iNOS is involved in the observed increase in capillary volume density. In other experimental models it has also been shown that stimulation of •NO production induces capillary-network formation and angiogenesis, and that L-NAME suppresses these effects (Ziche et al., 1994; Ando et al., 2002; Suzuki, 2006).

Besides acting as a vasodilatator and integrating/activating various angiogenic factors, •NO acts on the vascularization process by modulating expression of vasoconstriction factors. Kourembanas et al. (1993) have shown that •NO inhibits the expression of hypoxia-induced endothelin-1, whereas L-NAME increases its production 3 to 4 times.

In contrast to our results and earlier ones, Uchida et al. (1994) show that blood flow in IBAT has a short-term and dose-dependent increase after intravenous injection of L-NAME or N^G-monomethyl-L-arginine. Multiple mechanisms of •NO action in IBAT probably also depend on interactions with other parts of the redox system which can modulate its intracellular concentration, primarily on the degree of the reaction with $O_2^{\bullet-}$. Furthermore, L-NAME, as a competitive inhibitor of NOS, in addition to reducing monoxygenation of arginine with •NO production, also increases NOS dependent cytochrome c reductase activity, resulting in an increase of $O_2^{\bullet-}$ production (Bachschmid et al., 2005).

Marikovsky et al. (2002) emphasize the role of CuZnSOD in angiogenesis. Transgenic CuZnSOD mice show a three times higher angiogenic potential, whereas inhibition of CuZnSOD also inhibits their angiogenesis. In contrast, Wheeler et al. (2003) show that overexpression of extracellular SOD inhibits tumor vascularization in mice. These studies indicate that SOD can display either an angiogenic or antiangiogenic effect, depending on the isoforms and their localization. Results from the present study suggest that observed changes in CuZnSOD activity are part of an overall homeostatic response directed to angiogenic potential maintenance. Finally, the effect depends on the concentration of $O_2^{\bullet-}$ and its interaction with other redox molecules, primarily •NO. The $O_2^{\bullet-}$ is produced in all cells, concentrationally dependent on the cell type, metabolic state, and SOD activity. It is an antagonist of the physiological response of •NO. The rate constant for this reaction is threefold higher than between SODs and $O_2^{\bullet-}$ (Huie and Padmaja, 1993). In this context, •NO may act as an $O_2^{\bullet-}$ scavenger, decreasing the concentration of $O_2^{\bullet-}$. Considering that endogenous level of SOD depends on $O_2^{\bullet-}$ concentration (Fridovich 1978), depression of SODs activity could be a response to decreased $O_2^{\bullet-}$ availability. On the other hand, the product of the •NO and $O_2^{\bullet-}$ reaction is peroxynitrite ($ONOO^-$), which nitrates tyrosine residues in proteins (Padmaja et al., 1996).

In our previous *in vivo* study we showed that a chronic L-arginine treatment decreases the activity of MnSOD in IBAT of animals acclimated to room temperature, and suggested that the interaction between •NO and $O_2^{\bullet-}$ is significant in the mitochondrial function of the tissue (Petrovic et al., 2005). Furthermore, we showed that after a long-term cold-acclimation (45 days at 4°C), the markedly reduced MnSOD activity is probably a consequence of lower $O_2^{\bullet-}$ production in mitochondria, caused by intensified uncoupling. Many authors reported decreased $O_2^{\bullet-}$ production by uncoupling (Skulachev, 1994; Echtay et al., 2005). In the present paper, both uncoupling (long-term cold-acclimation) and L-arginine treatment (room temperature) are probably included in the adaptive decrease of CuZnSOD activity, lowering the intracellular concentration of $O_2^{\bullet-}$. As a confirmation of these hypotheses, the strongest immunoreaction for nitrotyrosine - $ONOO^-$ reaction product, was detected in L-arginine-treated rats acclimated to room temperature. Moreover, among groups kept at cold, nitrotyrosine immunopositivity was detected only in the L-arginine-treated group. L-arginine treatment in animals kept at room temperature enhanced both eNOS expression and nitrotyrosine immunopositivity (indicating intense •NO and $O_2^{\bullet-}$ interplay) while decreased CuZnSOD activity compared to control. On the contrary, in the L-arginine-treated cold acclimated group, faint eNOS and nitrotyrosine expression were observed, while there was not any change in CuZnSOD activity compared to the cold-acclimated control. These data explain why differences seen in CuZnSOD activity in control groups are lost in L-arginine treatment. L-NAME treatment, as far as CuZnSOD activity is concerned, attenuates the effect of L-arginine. It is possible that this effect is related to the pronounced nuclear localization of CuZnSOD in brown adipocytes. We have also shown previously a nuclear immunopositivity for iNOS in animals at room temperature, treated with L-NAME, but this correlation is only speculative at the moment (Petrovic et al., 2005).

Our results are in agreement with the studies reported so far, which indicate that •NO, in addition to modulating various functions in IBAT, also has a role in angiogenesis. Treatment with L-arginine enlarges the

capillary network in IBAT, and the contact between capillaries and brown adipocytes. The opposite effect of L-NAME confirms that. In addition, we suggest that the interaction between $^{\bullet}NO$ and $O_2^{\bullet-}$, as well as the adaptive regulation of CuZnSOD, have roles in the modulation of the angiogenic response in IBAT. The increased eNOS expression in both L-arginine-treated groups, accompanied by increased nitrotyrosine immunoreaction compared to corresponding controls, strengthens this hypothesis. However, additional experiments are needed to confirm the mechanisms suggested.

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References

Aherne W.A. and Dunnhill M.S. (1982). Morphometry. Edvard Arnold Ltd. London.

Ando A., Yang A., Nambu H. and Campochiaro P.A. (2002). Blockade of nitric-oxide synthase reduces choroidal neovascularization. Mol. Pharmacol. 62, 539-544.

Asano A., Kimura K. and Saito M. (1999). Cold-induced mRNA expression of angiogenic factors in rat brown adipose tissue. J. Vet. Med. Sci. 61, 403-409.

Asano A., Irie Y. and Saito M. (2001). Isoform-specific regulation of vascular endothelial growth factor (VEGF) family mRNA expression in cultured mouse brown adipocytes. Mol. Cell. Endocrinol. 174, 71-76.

Bachschmid M., Schildknecht S. and Ullrich V. (2005). Redox regulation of vascular prostanoid synthesis by the nitric oxide-superoxide system. Biochem. Biophys. Res. Commun. 338, 536-542.

Czapski G. and Goldstein S. (1995). The role of the reactions of NO with superoxide and oxygen in biological systems: a kinetic approach. Free Radic. Biol. Med. 19, 785-794.

Echtay K.S., Pakay J.L., Esteves T.C. and Brand M.D. (2005). Hydroxynonenal and uncoupling proteins: a model for protection against oxidative damage. Biofactors 24, 119-130.

Foster D.O. and Frydman M.L. (1978). Nonshivering thermogenesis in the rat. II. Measurements of blood flow with microspheres point to brown adipose tissue as the dominant site of the calorigenesis induced by noradrenaline. Can. J. Physiol. Pharmacol. 56, 110-122.

Fredriksson J.M., Lindquist J.M., Bronnikov G.E. and Nedergaard J. (2000). Norepinephrine induces vascular endothelial growth factor gene expression in brown adipocytes through a beta - adrenoreceptor/cAMP/protein kinase A pathway involving Src but independently of Erk1/2. J. Biol. Chem. 275, 13802-13811.

Fridovich I. (1978). Superoxide dismutases: defence against endogenous superoxide radical. Ciba Found Symp. 65, 77-93.

Fukumura D., Gohongi T., Kadambi A., Izumi Y., Ang J., Yun C.O., Buerk D.G., Huang P.L. and Jain R.K. (2001). Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc. Natl. Acad. Sci. USA 98, 2604-2609.

Huie R.E. and Padmaja S. (1993). The reaction of NO with superoxide, Free Radic. Res. Commun. 18, 195-199.

Kikuchi-Utsumi K., Gao B., Ohinata H., Hashimoto M., Yamamoto N. and Kuroshima A. (2002). Enhanced gene expression of endothelial nitric oxide synthase in brown adipose tissue during cold exposure. Am. J. Physiol. 282, R623-R626.

Kourembanas S., McQuillan L.P., Leung G.K. and Faller D.V. (1993). Nitric oxide regulates the expression of vasoconstrictors and growth factors by vascular endothelium under both normoxia and hypoxia. J. Clin. Invest. 92, 99-104.

Kuroshima A. (1993). Brown adipose tissue thermogenesis as physiological strategy for adaptation. Jpn. J. Physiol. 43, 117-139.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Ma S.W. and Foster D.O. (1984). Redox state of brown adipose tissue as a possible determinant of its blood flow. Can. J. Physiol. Pharmacol. 62, 949-956.

Marikovsky M., Nevo N., Vadai E. and Harris-Cerruti C. (2002). Cu/Zn superoxide dismutase plays a role in angiogenesis. Int. J. Cancer 97, 34-41.

Misra H.P. and Fridovich I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170-3175.

Nagashima T., Ohinata H. and Kuroshima A. (1994). Involvement of nitric oxide in noradrenaline-induced increase in blood flow through brown adipose tissue. Life Sci. 54, 17-25.

Padmaja S., Squadrito G.L., Lemercier J.N., Cueto R. and Pryor W.A. (1996). Rapid oxidation of DL-selenomethionine by peroxynitrite. Free Radical. Biol. Med. 21, 317-322.

Petrovic V., Korac A., Buzadzic B. and Korac B. (2005). The effects of L-arginine and L-NAME supplementation on redox-regulation and thermogenesis in interscapular brown adipose tissue. J. Exp. Biol. 208, 4263-4271.

Petrovic V., Buzadzic B., Korac A., Vasilijevic A., Jankovic A. and Korac B. (2006). Free radical equilibrium in interscapular brown adipose tissue: relationship between metabolic profile and antioxidative defense. Comp. Biochem. Physiol. C 142, 60-65.

Saha S.K., Ohinata H. and Kuroshima A. (1996). Effects of acute and chronic inhibition of nitric oxide synthase on brown adipose tissue thermogenesis. Jpn. J. Physiol. 46, 375-382.

Saha S.K., Ohno T., Ohinata H. and Kuroshima A. (1997). Effects of nitric oxide synthase inhibition on phospholipid fatty acid composition of brown adipose tissue. Jpn. J. Physiol. 47, 477-480.

Smith R.E. and Roberts J.C. (1964). Thermogenesis of brown adipose tissue in cold-acclimated rats. Am. J. Physiol. 206, 143-148.

Skulachev V.P. (1994). Decrease in the intracellular concentration of O2 as a special function of cellular respiratory system. Biokhimiia 59, 1910-1912.

Suzuki J. (2006). L-arginine supplementation causes additional effects on exercise-induced angiogenesis and VEGF expression in the heart and hind-leg muscles of middle-aged rats. J. Physiol. Sci. 56, 39-44.

Takada Y., Noguchi T., Okabe T. and Kajiyama M. (1982). Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. Cancer Res. 42, 4233-4235.

Uchida Y., Tsukahara F., Irie K., Nomoto T. and Muraki T. (1994). Possible involvement of L-arginine-nitric oxide pathway in modulating regional blood flow to brown adipose tissue of rats. Naunyn Schmiedebergs Arch. Pharmacol. 349, 188-193.

Wheeler M.D., Smutney O.M. and Samulski R.J. (2003). Secretion of extracellular superoxide dismutase from muscle transduced with recombinant adenovirus inhibits the growth of B16 melanomas in mice. Mol. Cancer Res. 1, 871-881.

Ziche M., Morbidelli L., Masini E., Amerini S., Granger H.J., Maggi C.A., Geppetti P. and Ledda F. (1994). Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. J. Clin. Invest. 94, 2036-2044.

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