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RESEARCH ARTICLE

COLD EXPOSURE AND ADIPOSE NITRIC OXIDE AND MAST CELLS: INFLUENCE ON AORTA CONTRACTILITY

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Abstract

Both nitric oxide (NO) and mast cells play important roles in adipose and vascular tissue biology. Chronic cold stress decreases the sensitivity of vascular smooth muscle to various contractile agents including norepinephrine (NE). In our previous cold exposure study we found that the contractile response of isolated rat aortas to NE was significantly reduced, and the number of rat aortic adventitial mast cells decreased. Histologically and functionally, white and brown adipose tissue (WAT and BAT) can be distinguished. Beyond its significance in energy store/release and heat production, adipose tissue secretes multiple signaling molecules that have endocrine and paracrine role in the regulation of vascular functions. The aims of the present study were to examine chronic cold exposure-induced alterations in (*i*) the concentration of NO released from selected regions of WAT and BAT in female and male rats, (*ii*) the histochemistry of white and brown adipose mast cells, and (*iii*) whether adipose-derived NO affects the contraction of isolated rat aorta to NE. Twelve females and 12 males Spraque-Dawley rats (150-200 g body weight) were used. The rats were exposed to a cold/freely moving stress for 2 hours each day for 5 consecutive days. At the end of cold exposure, the rats were sacrificed, and samples of thoracic aorta with associated periadventitial adipose tissue (*tunica adiposa*) were obtained. WAT and BAT were isolated from subcutaneous abdominal and interscapular areas, respectively. The concentration of NO was measured by capillary electrophoresis and mast cells were evaluated histochemically. The response of aorta smooth muscles to NE was recorded in the isolated organ bath. To determine whether adipose-derived NO affects aorta contraction to NE, cumulative dose response curves to NE (10^{-8}–10^{-3} M) were obtained with or without isolated WAT/BAT suspended in the organ bath medium. In control animals, a gender-related significant difference in NO production in both WAT and BAT was found, NO levels being significantly higher in female than male rats. Data from the contractile response of isolated aorta to NE suggest that receptor affinity to NE is significantly different between female and male controls. Presence of BAT and WAT (isolated from cold-exposure animals) in the bath changed the response of aorta smooth muscle to NE. Displaying a gender dimorphism, BAT/WAT-derived NO, or other vasorelaxing factors, seem to reduce receptor density and/or affinity to NE. Adipose mast cell histochemistry also showed diversity in respect to subtype, gender, and cold exposure. Altogether, we found (*i*) a gender difference in adipose-released NO and in adipose mast cell histochemistry to cold exposure, and (*ii*) peripheral adipose tissues affect aortic contractile responses to NE likely by a NO-mediated pathway during cold exposure, suggesting that adipose tissue may limit cold-induced excessive vasoconstriction. Our ongoing study aims at the evaluation of whether aortic *tunica adiposa* itself could also contribute to this phenomenon.

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Introduction

Adipose tissue has traditionally been recognized as the most important lipid/energy store and heat producer of the body. Moreover, recent studies clearly demonstrate that adipose tissue produces and releases - via endo- and paracrine way - a large number of singnaling proteins, collectively termed adipokines. These exert a variety of local, peripheral, and central effects, including the regulation of cardiovascular functions (1-9).

Generally, two different types of adipocytes are known in mammals: white adipocytes, which store energy as triglycerides and release it according to organism needs, and brown adipocytes, which dissipate energy as heat (4). Although a concept of adipocyte plasticity is recently emerged (4), morphologically and functionally two types, white and brown adipose tissue (WAT and BAT) are described.

Initiated by Soltis and Cassis in 1991 (5), recent progress in cardiovascular adipobiology increasingly demonstrates that periadventitial adipose tissue (*tunica adiposa*) (6) has profound paracrine effect on blood vessel contractility. For instance, adiposa-denuded vessels show reduced contractility to various agents such as norepinephrin (NE), angiotensin II, and phenylephrine, suggestive of a paracrine release of adipose-derived relaxing factor(s) (7-9). Therefore, it should be reasonable to assume that periadventitial adipose tissue, via paracrine way (reviewed in 6) and peripheral WAT and BAT, via endrocrine way, can regulate the arterial tone.

The activity of sympathetic nervous system is increased following cold exposure. This results in generalized vasoconstriction and skeletal muscle shivering to maintain body temperature. However, chronic cold stress, which is one of the stress paradigms having a dramatic effect on sympathetic nerves, has only a moderate pressor effect and decreases the sensitivty of vascular smooth muscles to various contractile agents including NE (10,11) . Hence, there may be a protective mechanism preventing the development of excess vasoconstriction during cold exposure even though sympathetic nerve activity is high.

It has been reported that nitric oxide synthase (NOS) gene expression is significantly higher when body temperature decreased (10). Both WAT and BAT express NOS isoforms (12). "Brown" NO release increases in cold-exposure as well as BAT weight increases in 2-3 fold during cold acclimatization (13-15).

Mast cells are widely distributed in all tissues including adipose tissue (16,17). These are multifunctional cell type, involving in the control of cardiovascular functions, tissue injury and repair, inflammation, thermogenesis, lipid metabolism, and obesity and diabetes (16-22); mast cells are "master cells", to paraphraze Stephen J. Galli (*N Eng J Med* 1993; 328:257-265). Mast cells synthesize and secrete a variety of mediators, including NO (12,15,19,20), which is also accepted as a major determinant of mast cell phenotype (23).

In our previous cold exposure study (11) we found a decrease in the number and degranulation of rat aortic adventitial mast cells. This study also revealed that NE-induced contractile response of isolated rat aorta was significantly reduced.

It is known that there are gender differences in body fat amount and distribution. Men have less body fat and a greater amount of abdominal adipose tissue than women of the same body mass index (24-26). In female rats, it was shown that BAT has higher oxidative and thermogenic capacities (27).

The aims of the present study were to examine chronic cold exposure-induced alterations in (*i*) the concentraction of NO released from selected regions of WAT and BAT in female and male rats, (*ii*) the histochemistry of white and brown adipose mast cells, and (*iii*) whether adipose-derived NO affects the contraction of isolated rat aorta to NE.

Material and methods

In the experiment, 12 females and 12 males Spraque-Dawley rats (150-200g body weight) were used. Rats were divided into four groups: controls (females and males) and cold-stressed (females and males).

In cold stress procedure, the rats were exposed to a cold/freely moving stress for 2 hours (from 8.00 AM to 10.00 AM) each day for 5 consecutive days. Three animals were put in a cage being able to move freely and then placed into cold chamber (+ 4° C). Rats in control groups were kept at room temperature. At the end of cold exposure, the rats were sacrificed by cervical dislocation and samples of thoracic aorta were obtained for measurement of isometric contractile force. Aortas were obtained with their associated periadventitial adipose tissue and cut into ring of approximately 2 mm length. In order to test the effect of NO released from peripheral adipose tissue, that is, WAT- and BAT-derived NO, a bioassay method was applied. Subcutaneous abdominal WAT and interscapular BAT were isolated from both control and cold-exposured female and male rats. For bioassay recording, WAT and BAT, in equal size and weight, was individually anchored into the organ bath medium via silk thread, and cumulative NE response were recorded. White adipose tissue and BAT from male were used for aortas isolated from male and *vice versa*.

Aorta rings with or without isolated BAT/WAT were mounted in organ chambers (Hugo–Sachs 4 container Schuler) filled with Krebs' solution of the following composition (in mM) 118.4 NaCl, 7.4 KCL, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.7 glucose at 37^{0}C and bubbled with 95%O_2 and 5% CO_2 (pH:7.4). Cumulative dose response curves to NE (10^{-8}-10^{-3} M)

were recorded by Biopac Data Acquisition system.

After mounting, each preparation was equilibrated for 45 min. After equilibration in physiological solution basal tension of the vessel was adjusted to 0.7g and the preparations were stimulated with a 100mM KCL depolarizing solution (11). After wash out and 30 min recovery vessels were exposed to cumulatively added NE (10^{-8}-10^{-3} M).

Determination of NO levels and mast cells histochemistry in adipose tissue

Isolated WAT and BAT samples were seperated for NO measurement and mast cell histochemistry. For mast cells histochemistry, formalin fixed samples were stained with alcian blue/safranin. Mast cells were categorized, as described in our previous reports, as mast cells with no-heparin (blue-stained cells) and mast cells with high heparin (red-stained cells) (11).

For NO measurement, tissue samples, first weighted and then stored in a deep freeze at -80°C until analysis time. Nitric oxide levels were measured by capillary electrophoresis (28). Fused-silica capillary was filled with the background electrolyte consisting of 200 mM lithium chloride, 10 mM borate buffer at pH 8.5. All of the solutions were prepared in a nitrate-free double distilled water. The dilutions of nitrate were made from the stock solutions of 1.06X 10^{-3} M KNO and 4.39X10^{-3} M KBr as internal standard (I.S.). The final concentration of I.S. was always 2.92X10^{-3} M in the calibration and sample solutions. The tissues were homogenized in a 0.5 ml phosphate buffer (PBS) and centrifuged in 5000 RPM for 5 minutes. 100 µl supernatant was taken and 200 µl KBR(ACN) added then centrifuged in 5000 RPM for 5 minutes. 200 µl supernatant was injected. The column was washed and conditioned by rinsing, in turn, 5 min each with 0.1 M NaOH, Besides, 2-min washing with background electrolyte was made between each of the experiments. The detection was made at 214 nm where monochromatic light is absorbed maximum by the related anions. The injection time was 50 ms (corresponds to almost 25 nl) using vacuum injection mode and reversed polarity controlled current of 200 µA corresponds to 12.7 kV was applied.

Statistical analysis

Data are expressed ± S.E.M. Analysis of contractile responses and pD_2 (apparent agonist affinity constant, -log EC_{50}) values were calculated using GraphPad Software V2.04. Statistical differences were evaluated using one way ANOVA followed by the Student Newman-Keuls test. Data of NO concentration of males and females were one-way analysis of variance (ANOVA) followed by Dunnett's. P<0.05 was taken as significant.

Results

Adipose-derived NO

Figure 1 shows NO levels of WAT and BAT of male rats. In control males, NO level of BAT is significantly higher than that of WAT. Cold exposure significantly increased NO levels of WAT and BAT compared to control levels of WAT. Figure 2 shows NO levels of WAT and BAT of female rats. Contrary to male, in control animals the levels of BAT- and WAT-released NO did not display any significant difference. In both types of adipose tissue, NO levels of female rats were not significantly changed

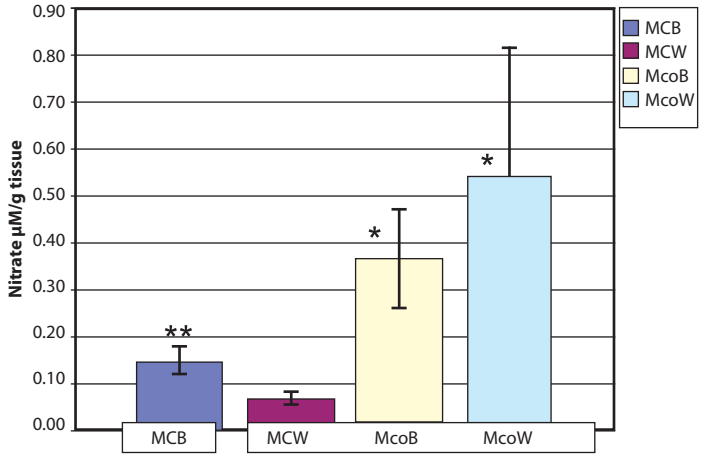

Figure 1. Nitric oxide levels in white and brown adipose tissues of male rats.
Male Control WAT (MCW) - Male Cold WAT (McoW)
Male Control BAT (MCB) - Male Cold BAT (McoB)
*P< 0.001 Significantly different from MCW group
**P<0.001 Significantly different from MCW group

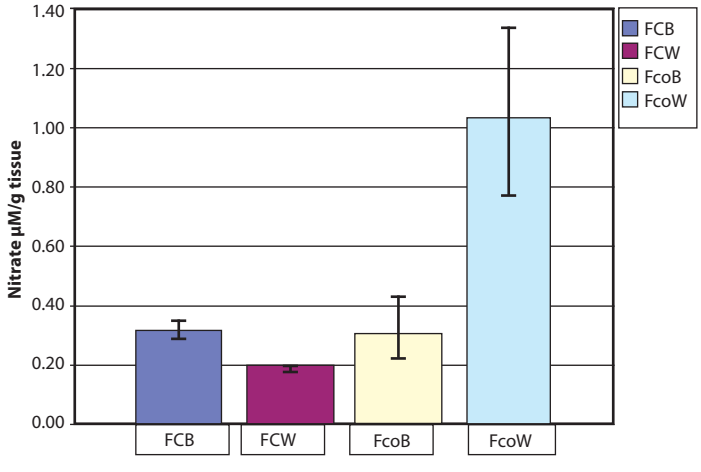

Figure 2. Nitric oxide levels in white and brown adipose tissue of female rats.
Female Control WAT (FCW) - Female Cold WAT (FcoW)
Female Control BAT (FCB) - Female Cold BAT (FcoB)

by cold exposure. Statistical comparison of male and female adipose NO levels are given in Figure 3 A, B. Nitric oxide levels of BAT and WAT of the control animals revealed significant differences between male and female rats. Both WAT and BAT of female animals have higher NO levels than males. Influence of cold lead to significant increase in NO levels of WAT in both sex compared to only male controls.

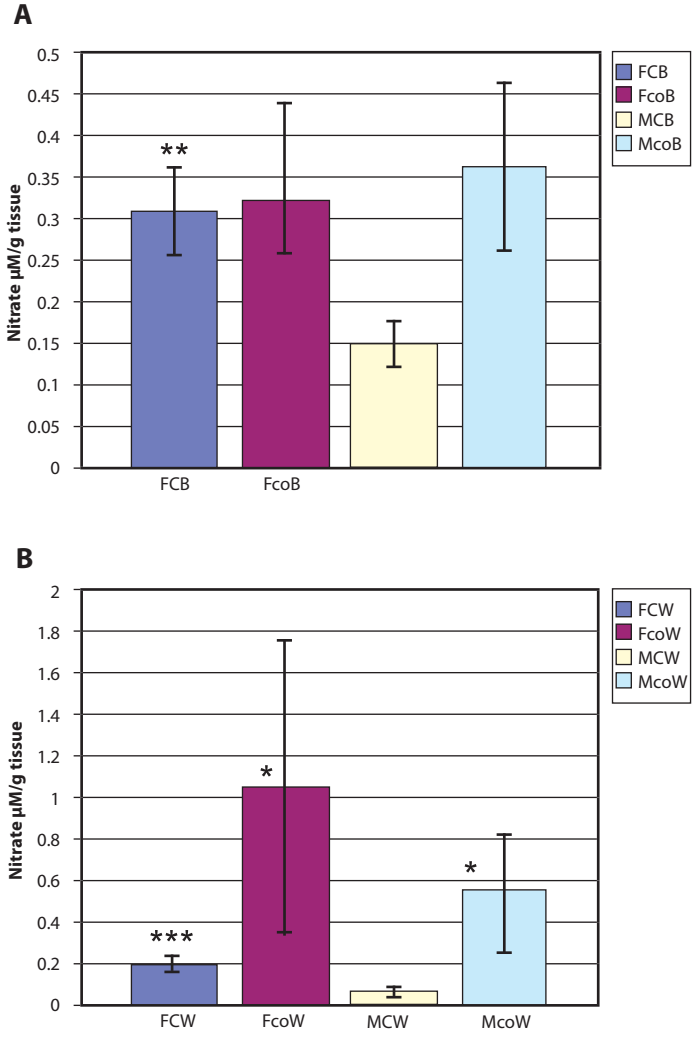

Figure 3 A, B. Comparison of NO levels of brown adipose tissue (A) and white adipose tissue (B) between female and male rats.

Male Control WAT (MCW) - Male Cold WAT (McoW)
Male Control BAT (MCB) - Male Cold BAT (McoB)
Female Control WAT (FCW) - Female Cold WAT (FcoW)
Female Control BAT (FCB)- Female Cold BAT (FcoB)
* P<0.05 Significantly different from MCW
** P<0.01 Significantly different from MCB
**** P<0.01 Significantly different from MCW

Contractile response of aorta

Figure 4 and Table 1 show the concentration-effect curves to NE and pD_2 (apparent agonist affinity constant, -log EC_{50}) values of male and female control rats, respectivelly. The NE dose-response curve for aorta of control male rats shifted to the right, producing a significantly higher pD_2 (-log EC_{50}) value without any significant reduction in the maximum response. The higher pD_2 values indicate reduced sensitivity of aortic smooth mus-

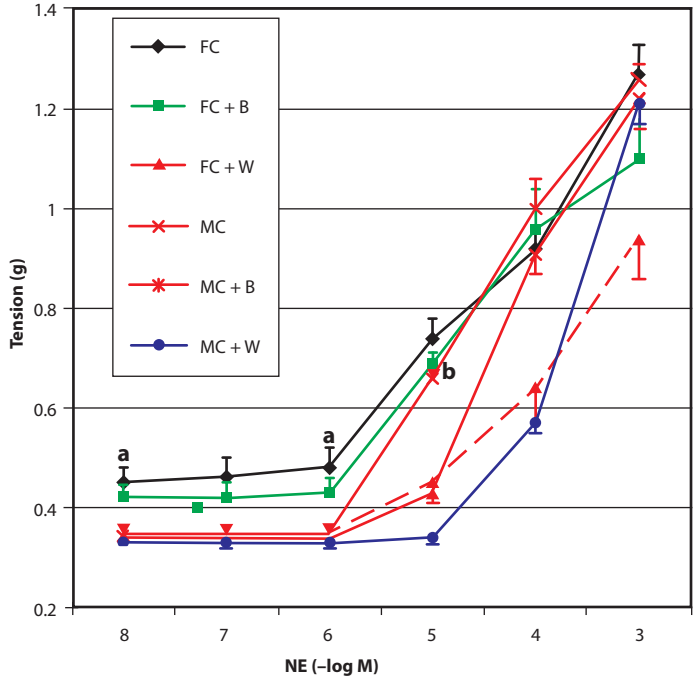

Figure 4. Mean concentration – effect curves to noradrenalin (NE) obtained in aorta rings isolated from male and female controls (MC and FC). +B and + W, with brown adipose and white adipose tissue in the bath medium, respectively.
a: Significantly different from MC, P<0.05
b: Significantly different from MC+W and FC+W, P<0.05

Table 1. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of male and female control rats. +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from MC group, P<0.05*
*** Significantly different from MC+W group, P<0.05*

	Group	PD_2 ± SEM	n
Female	FC	7.10 ± 0.21*	6
Female	FC+B	6.37 ± 0.19	6
Female	FC+W	6.52 ± 0.20**	6
Male	MC	6.45 ± 0.06	6
Male	MC+B	6.08 ± 0.05	6
Male	MC+W	5.79 ± 0.09	6

cle to NE in male rats. In other words, the sensitivity of aortic smooth muscle of male rats to NE was significantly less than that of female rats. Additionally more sensitivity reduction to NE in the aortic smooth muscle of male rats are observed when WAT, isolated from cold exposured rats, suspended into the bath medium. This response is in correlation with the increased NO levels in WAT isolated from cold-exposed male rats.

Figure 5 and Table 2 show the concentration-effect curves for NE and pD_2 values of aorta vessels isolated from cold exposure and control female rats, respectively. The response to NE (10^{-5}–10^{-3} M concentrations) of aorta of cold exposure female rats decreased but difference is not found statistically significant. The response to NE (10^{-8}–10^{-3} M concentrations) of aorta of cold exposure rats was significantly attenuated by suspending of BAT, isolated from cold exposure female rats, into the bath medium. Presence of BAT in the bath medium significantly increased the pD_2 values. However, NO levels of BAT of the female rats are not significantly changed by cold exposure. It may be suggested that besides NO, other BAT-derived molecule(s) may cause reducing the sensitivity of aortic smooth muscles to NE.

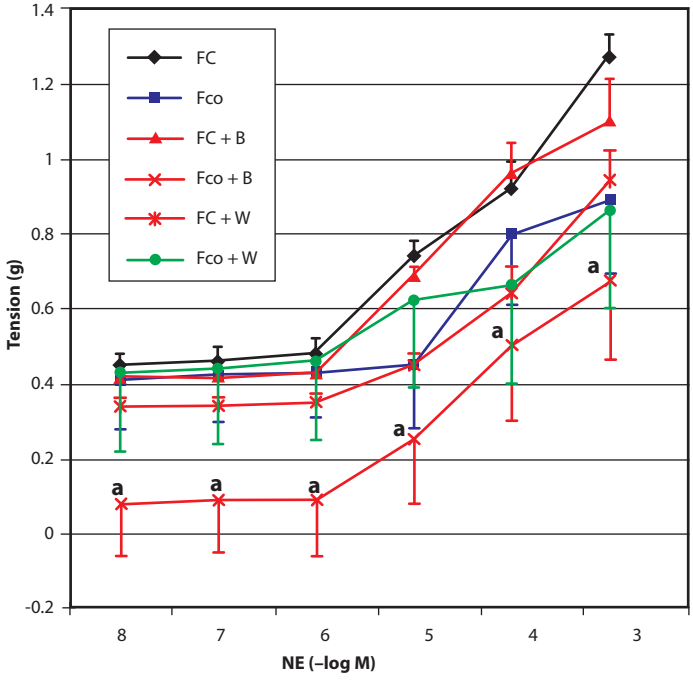

Figure 5. Mean concentration-effect curves to noradrenalin (NE) obtained in aorta rings isolated from control (C) and cold (co)-exposed female rats (F). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
a: Significantly different from all groups, P< 0.05.

Table 2. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of female rats (F). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from FC group, P<0.05*

Group	PD_2 ± SEM	n
FC	7.10 ± 0.21	6
Fco	6.14 ± 0.45	6
FC + B	6.37 ± 0.19	6
Fco + B	4.99 ± 0.47 *	6
FC + W	6.52 ± 0.20	6
Fco +W	5.80 ± 0.68	6

Figure 6 and Table 3 show the concentration-effect curves to

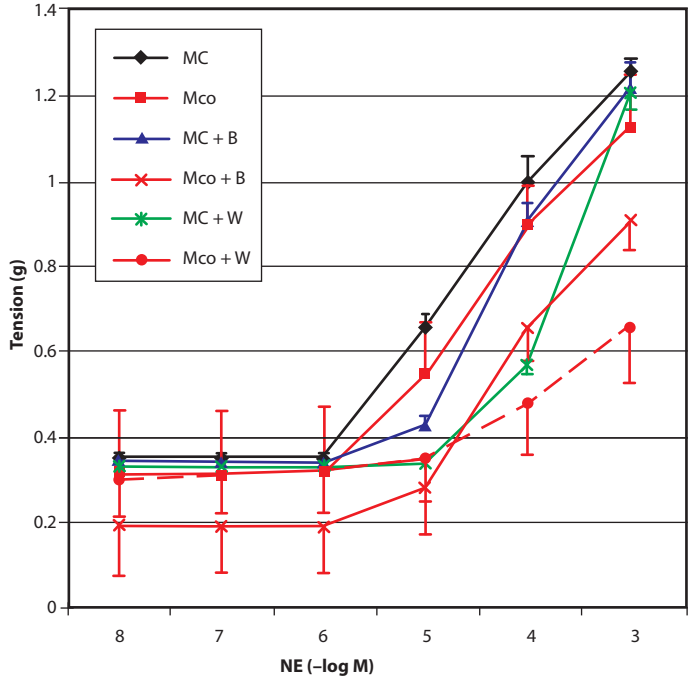

Figure 6. Mean concentration-effect curves to noradrenalin (NE) obtained in aorta rings isolated from control (C) and cold (co)- exposured male rats (M). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
a: Significantly different from MC and Mco, P< 0.05

Table 3. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of male rats (M). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from MC, Mco, MC+W groups, P<0.05*

Group	PD_2 ± SEM	n
MC	6.45 ± 0.06	6
Mco	5.63 ± 0.43	6
MC + B	6.08 ± 0.05	6
Mco + B	5.29 ± 0.30	6
MC + W	5.79 ± 0.09	6
Mco +W	4.44 ± 0.61*	6

NE and pD_2 values of aortas isolated from cold exposure and control male rats, respectively. Likewise, in female rats the response to NE for 10^{-5}–10^{-3} M concentrations of the aorta of cold exposured male rats decreased but difference is not found statistically significant. However, when WAT and BAT anchored into the organ bath medium, the contractile response of aortas obtained from cold exposured male rats significantly reduced to all concentration of NE (10^{-8}–10^{-3} M) as compared to the vessels of control and cold-exposed rats. WAT but not BAT causes a significant difference in pD_2 values (Table 3). WAT significantly reduced the sensitivity to NE of aortic smooth muscle isolated from cold exposured male rats. This response is in correlation with increased NO levels of WAT isolated from cold exposured male rats.

Mast cells

We found that BAT displayed more mast cells than WAT in both sex groups. The number of mast cells apprently reduced in both BAT and WAT after cold exposure, possibly resulting from an increased degranulation of these cells. Mast cells stained with alcian blue-safranin also showed different histochemistry according to types of adipose tissues, gender, and cold exposure. While mast cells stained red, blue and mixt in BAT of female rats, which means that mast cells contain heparin together with amines, male rats have only blue-stained mast cells, meaning that heparin can not be detected in the granules of the cells (Fig. 7B). After cold exposure, a few mast cells were detected in WAT and BAT of both sex groups. These mast cells increased heparin content in their granules and stanid with safranin in red (Fig. 7C).

Discussion

In the present study, a gender difference in adipose-released NO is observed. Our *in vitro* bioassay results indicate that aortic contractile response to NE and the effect of WAT- and BAT-derived molecules on the vessels reactivity are also different in male and female rats. Intriguingly, NO, or other vasorelaxing molecules, released from WAT more significantly affect the response of aortas of male rats, whereas molecules released from BAT more significantly affect the response of aortas of female rats. Such a gender as well as white-to-brown adipose difference are difficult to be explained.

We suggest that adipose-derived NO could be a key molecule to rescue the extreme cold-induced vasoconstriction. Nitric oxide also acts as a deactivator agent for NE (29). The NO system, including NOS and its endogenous inhibitor, asymmetric dimethylarginine, is involved in the control of thermogenic function of BAT and lipid metabolism in WAT (4,12,15,17,30-

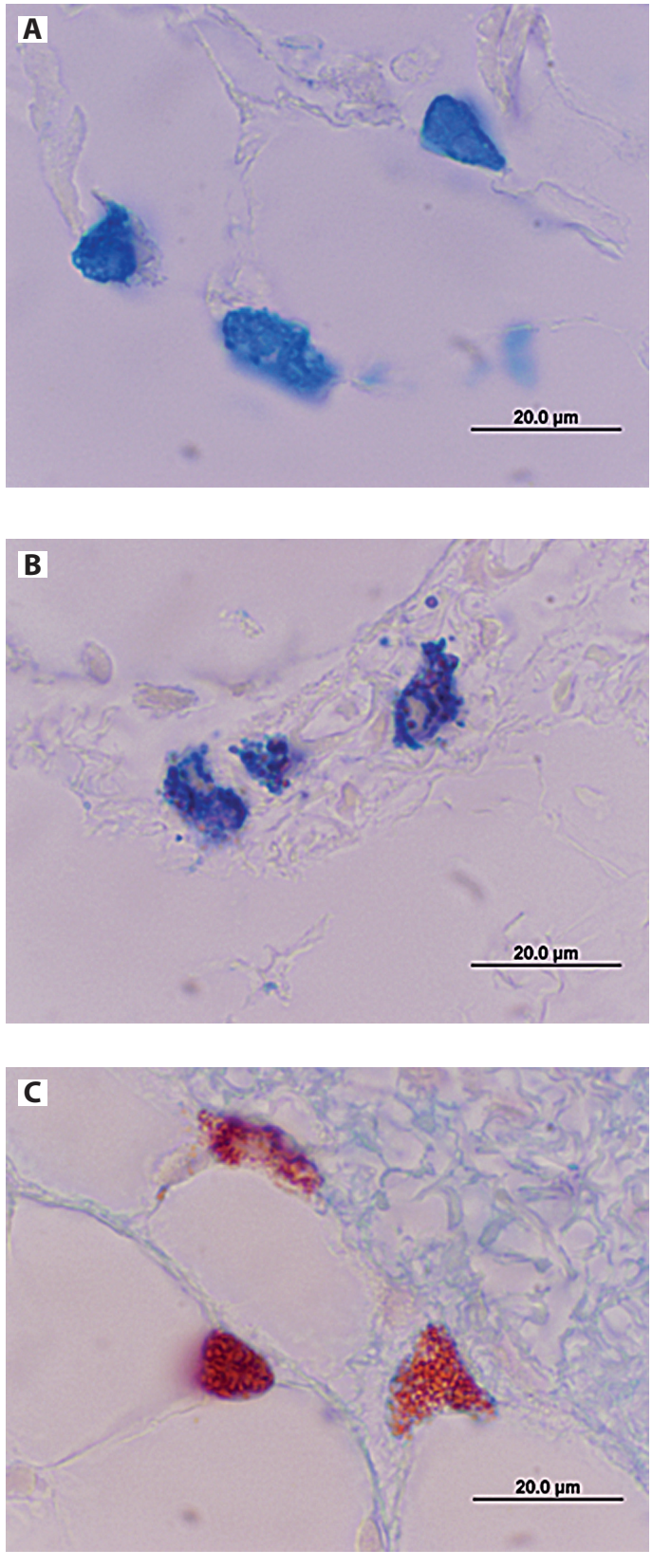

Figure 7. Blue stained mast cells in BAT of male rats (A); Mix stained mast cells in BAT of female rats (B); Red stained mast cells in WAT of cold-exposed male rats (C).

35). Cold exposure generaly diminishes both iNOS immunopositivity and protein levels in intrascapular BAT (35). It was suggested that iNOS may be involved in induction of apoptosis in this tissue. Because of the diminished iNOS activity, intrascapular BAT mass significantly increase in animals acclimated to cold (35). In the present study, cold stress did not significantly change NO levels of BAT isolated both male and female rats possibily by a decreased iNOS activity. On the other hand, cold-induced NO production is remarable in WAT in both sex groups. Further, cold exposure induces a decrease in the number of mast cells as well as changes in their granular content; it is difficult to extrapolate these results to a possible involvement of these cells in adipose tissue-released NO.

Conclusion

A gender difference in NO release and mast cell number and histochemistry in both WAT and BAT to chronic cold exposure of rats is found. Such a difference is also revealed in the contractile response of isolated aortas to NE. We may only speculate that estrogen-induced NO release (36) may somehow be involved in the observed gender difference in our study. It is possible that adipose-released NO may, at least in part, be responsible for the diminished contractile response of aortas to NE during cold exposure; this may limit cold-induced excessive vasoconstriction. Our ongoing study aims at the evaluation of whether aortic periadventitial adipose tissue-derived NO (37,38, cf. 39) and associated mast cells (16) and lympocytes (40) could also influence aortic contractility during cold exposure. In perspective, recently discovered periadventitial adipose tissue-derived vasorelaxants such as adiponectin (41,42), hydrogen sulfide (43,44) and angiotensin 1-7 (45, cf. 46) should be studied in cold exposure experiments. This may also be the case for human BAT (47-49).

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