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Normalization of aortic function during arousal episodes in the hibernating ground squirrel

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Abstract

Hypothermia is commonly used to restrict organ damage during preservation of tissue, but does not offer complete protection. Organ damage after reperfusion/rewarming is amongst others caused by an impairment of vascular properties, particularly endothelium-dependent vasodilatation. We hypothesized that hibernating small animals, which frequently cycle through periods of deep cooling (torpor) and full rewarming (arousal), employ specific mechanisms to preserve vascular function after cooling and reperfusion. Therefore we measured contraction of aortic tissue of hibernating European ground squirrels after 24 h and 7 days of torpor, arousal (1.5 h) and in non-hibernating animals. To assess the role of nitric oxide (NO), experiments were performed in the absence and presence of the NO-synthesis inhibitor, L-NMMA (10^{-4} M). Maximum contraction to phenylephrine and angiotensin II was doubled in 7-days torpid animals without a shift in EC_{50}, compared to the other 3 groups. Maximum contraction to KCl was doubled in 7-days torpid animals compared to the arousal group and non-hibernating animals. Relaxation to acetylcholine (ACh) and sodium nitrite in phenylephrine precontracted rings did not differ between groups. In the presence of L-NMMA, the maximum of

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concentration-response curves for all three vasoconstrictors was increased by about 30% in the arousal group, but unaffected in other groups. L-NMMA completely inhibited ACh-induced relaxation in 24-h torpid animals and non-hibernating animals, but only partially in 7-days torpid animals and in the arousal group. From this we conclude that vascular adaptation proceeds during torpor. Further, increased contractility of aortic tissue during long torpor returns to normal within 1.5 hours of arousal, which is associated with an increased basal NO synthesis. In addition, involvement of NO in agonist-mediated relaxation differs between the various stages of hibernation.Thus, hibernating animals have effectively developed mechanisms to preserve vascular function after cooling and rewarming. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Angiotensin II; Nitric oxide; Hibernation; Phenylenephrine; *Spermophilus citellus*

Introduction

Hypothermia is a commonly used method to restrict organ damage during the preservation of tissue. However, it does not provide complete organ protection. Especially during rewarming and reperfusion, organs are susceptible to damage. Changes in vascular function during the rewarming-reperfusion phase represent an important causative factor in the organ damage [1,2]. Studies in whole organs of non-hibernating mammals including heart, lung, liver, kidney and striated muscle show that rewarming after a prolonged period of deep cooling results in a severe inhibition of vasodilator response, with little or no impairment of vasoconstrictor properties [3–14]. Similar effects of cooling have been observed in studies using isolated arteries of non-hibernators [14,15].

Hibernating animals have developed strategies to limit organ damage induced by cooling and rewarming. This is particularly the case in small hibernating animals such as the European ground squirrel, as they frequently cycle through periods of deep cooling (torpor phase) and full rewarming (arousal phase) during hibernation. In these animals, major changes in cardiovascular performance are observed during the torpor phase, which are readily reversed during arousal without evident impairment of organ function [16]. However, the adaptations in vascular function during the different phases of hibernation are not well studied.

Changes in the functional properties of arterial tissue of small hibernating animals that occur during the torpor phase are well documented. Arteries from torpid animals show increased responsiveness to exogenous vasoconstrictor, without significant changes in vasodilator properties when compared to tissue from non-hibernating animals [17–20]. However, little is known about changes in vascular function during the torpor phase and upon arousal [17]. Because peripheral resistance and cardiac output normalize rapidly during arousal [16], it is conceivable that vascular function is quickly adjusted to offset the increased vasoconstriction observed during torpor. It is unknown whether this is dependent on recruitment of a compensating vasorelaxing mechanism, such as increased nitric oxide (NO) production. Moreover, it is not known whether normalization of vascular function during arousal depends on a systemic or a local factor.

The objective of the present study was to examine changes in vascular function of arterial tissue during different phases of hibernation in European ground squirrel (*Spermophilus citellus*). Therefore, we compared vasoconstrictor and vasodilator responses of aortic rings obtained from hibernating animals after short and long periods of torpor (24 h and 7 days, respectively) and after arousal (1.5 h), and from non-hibernating control animals. To assess the contribution of NO production, experiments were performed in the absence and the presence of the NO-synthase blocker, L-NMMA.

Methods

Animals

The study was approved by the Animal Experiments Committee of the University of Groningen (BG02198). The European ground squirrels *(Spermophilus citellus)* used were captured near Vienna [21], born in the laboratory from females that were caught pregnant or bred in the enclosures in Haren, the Netherlands. The animals were kept in lucite cages ($l \times w \times h = 48 \times 28 \times 50$ cm) with a nestbox attached ($l \times w \times h = 15 \times 15 \times 15$ cm). Wood shavings were used as bedding material, food (rabbit breeding chow, Teurlings, Waalwijk, the Netherlands) and water were supplied *ad libitum*. The animals were kept in a climate-controlled room at a relative humidity of 60 % throughout the experiment. Hibernation was induced by gradually lowering ambient temperature from 20 °C to 5 °C and changing light conditions from light:dark = 12:12 h to continuous dim red light (< 1 lux) during a 5-day period. Individual torpor-arousal patterns were assessed by measuring nestbox temperatures every minute with a computer based recording system [22]. Furthermore, six squirrels were equipped with a customized abdominal temperature logger (Fidbit, Onset, USA) to register body temperature every 48 min.

Four groups of animals were studied: (1) hibernating animals at low body temperature for 24 h (torpor short group; n = 6), (2) hibernating animals at low body temperature for 7 days (torpor long group; n = 5), (3) hibernating animals who were in arousal for 1.5 h after a 7 days torpor period (arousal group; n = 5) and (4) non-hibernating animals (n = 8). Arousal was induced by handling the animals for 3 min. Experiments in hibernating animals were carried out at least 10 weeks after the induction of hibernation. Averaged over all groups, the duration of their previous spontaneous torpor bout was 11.2 ± 0.4 days, and the length of their previous spontaneous arousal episode was 20.6 ± 0.9 hours. At the time of the experiment, the duration of torpor did not differ between 7-days torpid and aroused animals (7.0 ± 0.2 days and 7.1 ± 0.1 days, respectively). In the arousal group, the duration of arousal at the moment of removal of the aortic tissue was 1.5 ± 0.1 hour. Non-hibernating animals were studied 6–7 days after cessation of hibernation in spring by increasing ambient temperature to 25 °C. Measurement of body temperature at the moment of removal of the aorta confirmed that the groups represented the specific phases of hibernation. Rectal temperature amounted 30.9 ± 3.6, 8.0 ± 0.5, 8.2 ± 0.6 and 36.5 ± 1.3 °C for the arousal, 24-h torpor, 7-days torpor and non-hibernating group, respectively.

Aortic contraction measurement

Abdominal aortic tissue was removed from thiopental anesthetized animals (120 mg i.p.). The tissue was transported immediately to the laboratory in a Krebs bicarbonate solution of the following composition (mM): 120.4 NaCl, 5.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11.5 glucose and 25 $NaHCO_3$ and gassed with 95% O_2-5% CO_2. Upon arrival, the aorta was cleaned from fat and connective tissue and dissected into rings of approx. 3 mm width. During transport and dissection, rings were kept at a temperature of 4 °C (torpor groups) or 21 °C (arousal and non-hibernating group). Four rings of each animal were set up for recording of isotonic tension in a continuously gassed organ bath at the temperature of transportation, and were allowed to equilibrate for 60 min under a resting tension of 1.5 g still at 4 °C or 21 °C. After this period, bath solution of rings from torpid animals was gradually increased from 4 °C to 37 °C at a rate of 1 °C per 3 min. Rings at 21 °C were allowed an initial 51 min of equilibration, after which temperature was increased in a similar manner. Thus, total equilibration time was the same for rings from all groups. During equilibration from 21 °C to 37 °C, rings from all groups were contracted 3 times with high potassium Krebs solution (90 mM K^+, by replacing NaCl with KCl).

Experimental protocol

Vasoconstrictor properties were determined by obtaining subsequent, cumulative concentration-response curves for l-phenylephrine (phenylephrine; 10^{-9} – 10^{-4} M), KCl (5.6 – 90 mM) and angiotensin II (Angiotensin II; 10^{-10} – 3×10^{-6} M). Vasorelaxing properties were determined by obtaining cumulative concentration-response curves for acetylcholine (ACh; 10^{-8} – 10^{-4} M) and sodium nitrite (SN; 10^{-5} – 10^{-2} M) in rings pre-contracted by a sub-maximal concentration of phenylephrine (3×10^{-7} M). In between concentration-response curves, rings were washed 5 times and equilibrated at base-line level for 30 min. Experiments were performed in parallel rings in the absence or presence of L-NMMA (10^{-4} M). In rings treated with L-NMMA, the drug was added after the initial equilibration period and was continuously present thereafter.

Materials

Krebs buffer and drug solutions were freshly prepared daily. All compounds were from Sigma (St. Louis, MO, USA), except acetylcholine (Aldrich-Chemie, Steinheim, D), sodium nitrite (E. Merck, Darmstadt, D) and angiotensin II (University Hospital Pharmacy, Groningen, NL).

Statistics

Results are presented as mean ± s.e.m. Differences between concentration-response curves were analyzed using repeated measurement ANOVA (SigmaStat 1.01, Jandel Scientific, D).

Differences in other variables were tested using Student t-test. Differences were considered significant at $p < 0.05$.

Results

Daily temperature registration confirmed the presence of torpor/arousal patterns under laboratory conditions (Fig. 1). Aortic vascular function was assessed in rings from hibernating animals after 1.5 hours of arousal, from hibernating animals after 24 h and 7 days of torpor and from non-hibernating animals. All experiments were performed at 37 °C after a standardized rewarming protocol (see methods section).

Aortic contraction and relaxation during hibernation

Contractile properties of aortic rings were investigated by constructing cumulative concentration-response curves for phenylephrine, KCl and angiotensin II. Phenylephrine and Angiotensin II induced contractions (Fig. 2A-C) did not differ between rings from 24-h torpid, aroused and non-hibernating animals. In contrast, aortic rings from 7-days torpid animals showed a significant increase in the response to these agonists compared to the other groups, resulting in an about doubling of maximum contraction without a shift in EC_{50} (Fig. 2A,C). Maximal contraction to KCl was also doubled in the 7-days torpor group as compared to aroused and non-hibernating animals, whereas 24-h torpid animals showed an intermediate response compared to these groups (Fig. 2B).

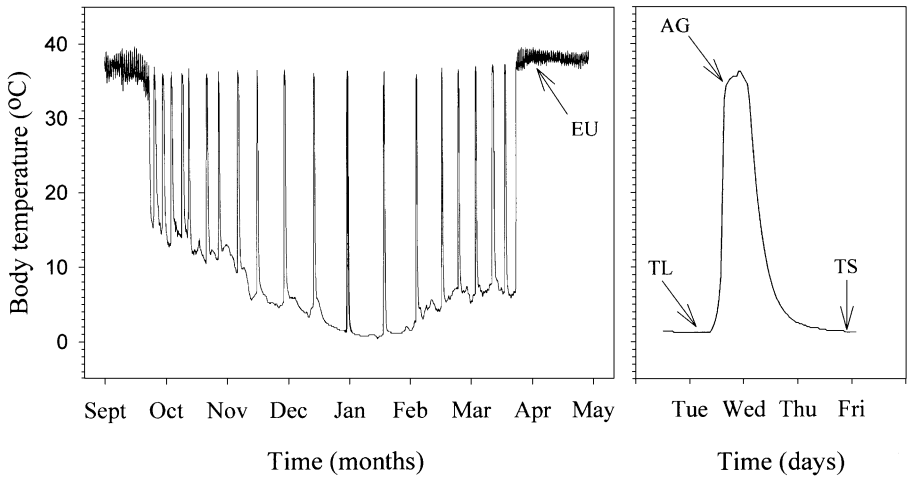

Fig. 1. Typical registration of the abdominal temperature by an implanted temperature logger, demonstrating the torpor/arousal pattern during hibernation under laboratory conditions in the European ground squirrel (left panel). Right panel: detail of the arousal period around January 1st (right panel). Arrows indicate the time points at which aortic tissue was obtained. TL = 7 – days torpor group, AG = arousal group, TS = 24-h torpor group, EU = non-hibernating animals.

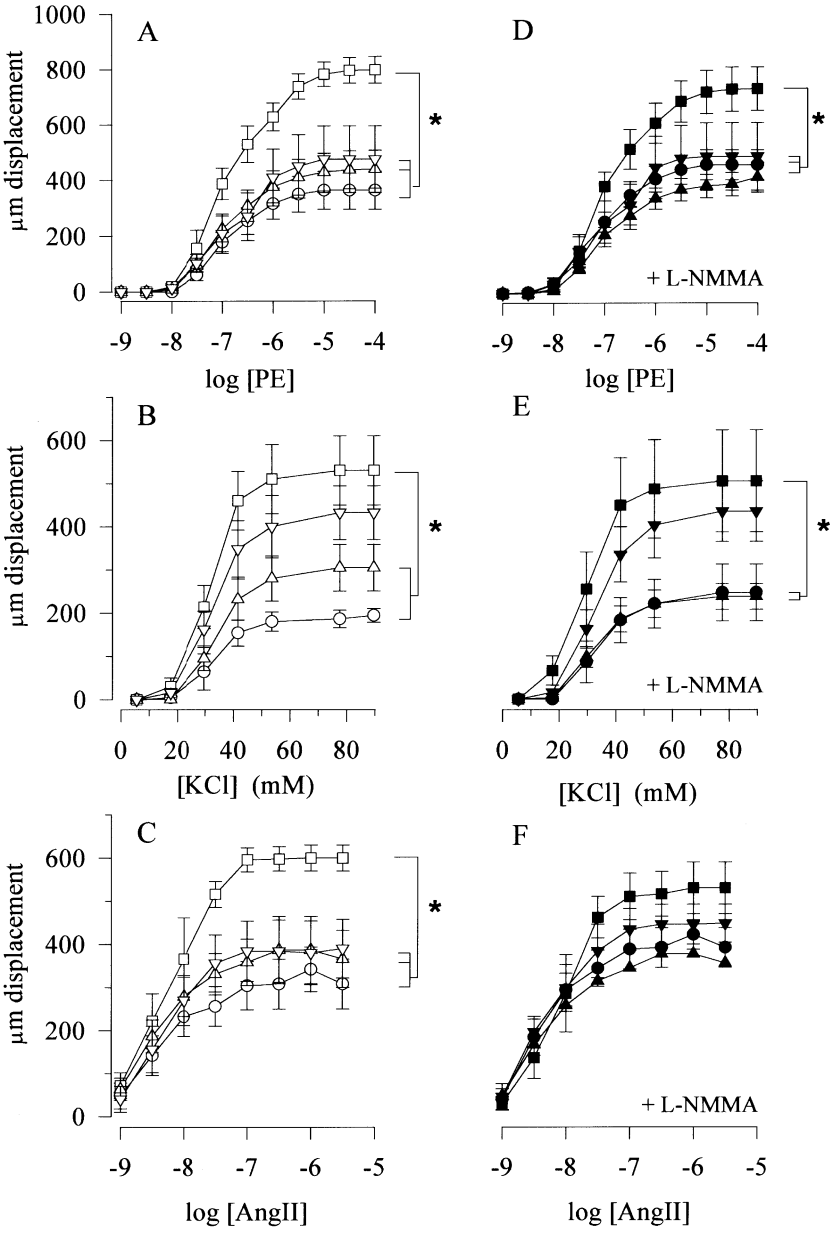

Fig. 2. Cumulative concentration-response curves for the contraction of aortic rings from animals after 24-h torpor (▽,▼; n = 6), 7-days torpor (□,■; n = 5), after 1.5 h in arousal (○,●; n = 5) and in non-hibernating animals (△,▲; n = 8) in response to phenylephrine (PE), KCl and angiotensin II (AngII). Curves were obtained in parallel rings in the absence (open symbols) or presence of L-NMMA (10^{-4} M, solid symbols). * = $p < 0.05$.

Endothelium-dependent and endothelium-independent relaxing properties of aortic rings were tested by obtaining cumulative concentration-response curves for acetylcholine (ACh)

and sodium nitrite (SN) in rings precontracted with phenylephrine (3×10^{-7} M). Concentration-response curves for ACh or SN did not differ between the groups (Fig. 3A,B). Thus, the increased contraction observed in the torpor group was normalized during arousal, whereas relaxing properties were similar in all groups.

Effects of blocking NO synthase on vascular responses

Involvement of the NO pathway was studied by performing the previously described experiments in parallel rings in the presence of the NO-synthase blocker, L-NMMA (10^{-4} M).

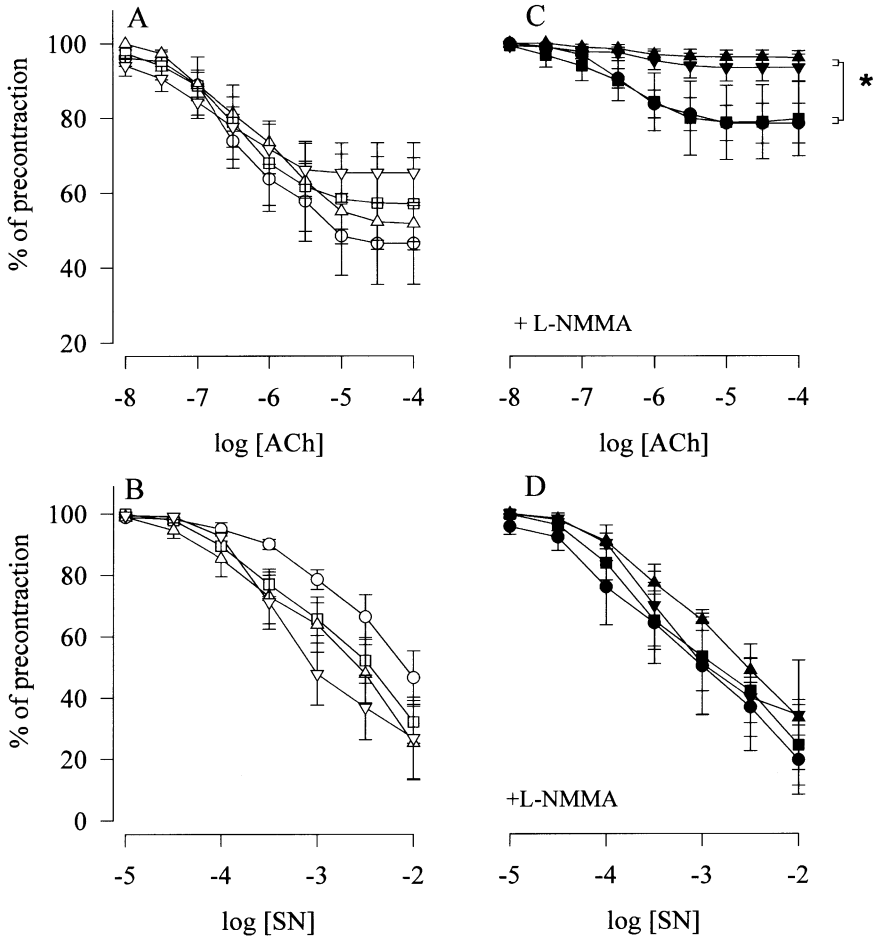

Fig. 3. Cumulative concentration-response curves for the relaxing effect of acetylcholine (ACh) and sodium nitrite (SN) of aortic rings from animals after 24-h torpor (▽,▼; n = 6), after 7-days torpor (□,■; n = 5), after 1.5 h in arousal (○,●; n = 5) and in non-hibernating animals (△,▲; n = 8). Curves were obtained in parallel rings precontracted with phenylephrine 3.10^{-7} M, in the absence (open symbols) or presence of L-NMMA (10^{-4} M, solid symbols). * = $p < 0.05$.

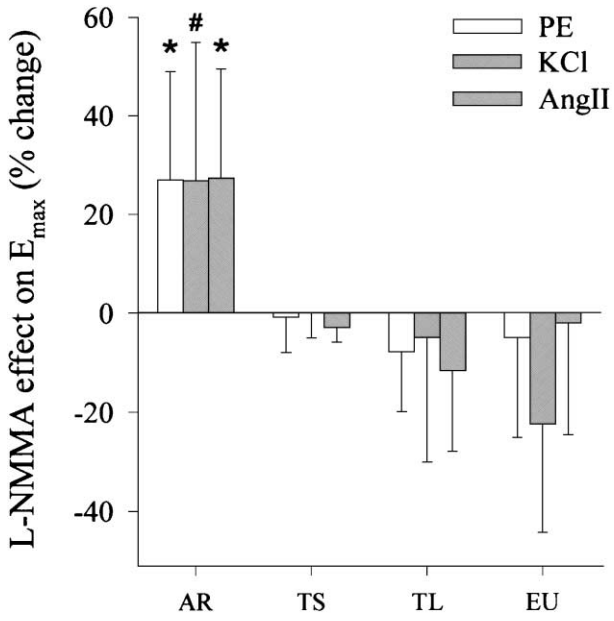

Fig. 4. The action of the NO synthase inhibitor L-NMMA (10^{-4} M) on the maximum effect obtained after cumulative concentration-response curves for phenylephrine (PE), KCl and angiotensin II (AngII). Experiments were performed in parallel rings in the absence and presence of L-NMMA (10^{-4} M) obtained from animals after arousal (AR, 1.5 h, n = 5), after 24-h torpor (TS, n = 6), after 7-days torpor (TL, n = 5) and from non-hibernating animals (EU, n = 8). L-NMMA significantly increased the maximum effect in rings from animals in arousal (1.5 h), but did not change the response in rings from torpid animals or non-hibernating animals. * = $p < 0.05$ compared to all other groups, # = $p < 0.05$ compared to TS, paired Student t-test.

In the arousal group, the maximum of the concentration-response curves for the vasoconstrictors phenylephrine, KCl and angiotensin II was increased by about 30% in the presence of L-NMMA (Fig. 4, 2D-F). In contrast, L-NMMA did not change the maximum contractile response to any of the agonists in rings from torpid and non-hibernating animals (Fig. 4, 2D-F). With respect to vasorelaxations, L-NMMA completely inhibited ACh-induced relaxation in phenylephrine-precontracted rings from 24-torpid and non-hibernating animals (Fig. 3A,C). However, responses were only partially blocked in rings from the 7-days torpor and arousal group (Fig. 3A,C). Further, relaxation to SN was unaffected by L-NMMA in all groups (Fig. 3C,D).

Discussion

This study shows an extensive regulation of vascular function in the hibernating European ground squirrel. The main findings are an increased contractile response of aortic rings in rings from 7-days torpid animals, returning to normal within 1.5 hours of arousal, which was associated with an increased basal NO synthesis in the arousal group. In addition,

endothelium-dependent relaxation is similar in all groups, but the contribution of NO to the relaxation is reduced after 7 days of torpor and in arousal.

This study demonstrates that changes in vascular function occur during the torpor phase, by comparing vascular responsiveness after different lengths of the torpor bout, i.e. 24 h and 7 days. Both contraction and relaxation differed after short and long torpor. The 7 days torpor group displayed an increased contractile response to phenylephrine and angiotensin II, which is primarily mediated via the $InsP_3/Ca^{2+}$ pathway. However, the response to KCl, mediated primarily via depolarization and subsequent activation of voltage-operated Ca^{2+}-channels, did not differ between both torpor groups. These observations suggest that the regulation of various signal transduction routes depends on the length of the torpor bout. In addition, L-NMMA completely blocked ACh-mediated relaxation after short torpor, whereas it was only partially effective after long torpor. Therefore, our results clearly demonstrate that torpor bout duration affects vascular responsiveness. However, it remains unclear whether changes during torpor are due to active regulation or reflect adaptations upon rewarming after different periods of cellular stress.

The increased response to phenylephrine as observed in aortic rings from 7-days torpid European ground squirrels is in accordance with the increased sensitivity to norepinephrine described in different arterial tissues from other hibernating species obtained at the torpor phase [18–20]. Also, the perseverance of basic relaxing properties to ACh during torpor (i.e. in the absence of L-NMMA) is consistent with findings in the mesenteric artery of the golden hamster [17,18]. Thus, the functional properties of aortic tissue of the European ground squirrel obtained at the torpor phase of hibernation resembles those of distinct arteries obtained in the other species studied, including woodchucks [20], thirteen-lined ground squirrel [19] and hamster [18].

This is the first study demonstrating functional changes in the vascular NO pathway during torpor and arousal, as observed using the NO-synthesis blocker L-NMMA. L-NMMA increased the maximum vasoconstriction in response to various agonists employed in the arousal group, but not in torpid and non-hibernating animals. In addition, L-NMMA completely abolished the relaxation to ACh in 24-h torpid and non-hibernating squirrels but only partially in the other groups.

The increase in contraction in presence of L-NMMA is generally thought to be due to loss of basally released NO [23]. Such view would be compatible with the observation that within each group L-NMMA affected the contraction of all three vasoconstrictors similarly. Therefore, results with respect to contraction suggest that basal NO release is increased in the arousal phase, but rapidly returns to control levels during torpor. This functional observation is in agreement with the increase in immunoreactivity for endothelial NO synthase found in the hamster renal artery during arousal [24]. In addition, vasorelaxation to ACh in 24-h torpid and non-hibernating squirrels was completely blocked by L-NMMA, thus suggesting total dependency on NO activity. In contrast, L-NMMA only partially blocked ACh-induced relaxation in 7-days torpid and aroused animals, indirectly implying the active presence of additional relaxation mechanisms (e.g. prostanoids, EDHF). From this, it is tempting to speculate that stimulated NO-activity may be decreased during these phases and that other pathways are recruited or become active to preserve general vasorelaxing

properties. When interpreted like this, our combined data further suggest that L-NMMA differentially affects the NO component involved in contraction and relaxation phases of hibernation. Particularly the increase in basal NO in arousal seems to be at odds with the "loss" of agonist (ACh)-evoked NO in the same animals. It should be noted, however, that important differences exist between mechanisms responsible for basal and stimulated NO, and even between stimulated NO after receptor-dependent and –independent stimulation [25]. For that matter, discrepancies between basal and stimulated NO production might be accounted for by different NO-synthase isoenzymes.

Our study shows that natural rewarming during 1.5 h (i.e. arousal) normalizes the increased contractile response as observed in aortic tissue of 7-days torpid animals. So far, only a study of Ralevic et al. described changes in contractile response during arousal in the mesenteric artery of the hibernating hamster [17]. In both species, the changes in vascular responsiveness during torpor are rapidly and completely reversed during the arousal episode. However, considerable differences exist in the particular changes. We found a rapid normalization of contraction during arousal to all agonists employed, i.e. phenylephrine, KCl and angiotensin II. In contrast, Ralevic et al. (1998) report an increase in response to exogenous norepinephrine in aroused animals as compared to torpid and control animals, while response to KCl was similar in all groups. Differences in animal species and – perhaps even more importantly – the nature of the arterial preparation may account for the dissimilarities between these results.

The main functional defect induced by prolonged cold storage of organs and isolated arteries is the (nearly) complete deterioration of endothelium-dependent relaxing properties [2–4, 9–11, 13–15] possibly due to impaired endothelial NO production [3,5]. Our study suggests that hibernating squirrels have resolved this problem, as in the absence of L-NMMA the ACh-induced relaxation was similar in torpid and aroused animals as compared with non-hibernating squirrels. In view of the above, several mechanisms appear to be involved in maintaining the relaxing properties throughout hibernation. First, the deterioration of NO-mediated relaxation is blunted in hibernating animals as compared to cold-stored arteries. Additionally, the NO-mediated relaxation seems to recover during arousal, as the ACh-induced relaxation in 24-h torpid animals was completely mediated by NO. Possibly, alternative signaling pathways mediating vasorelaxation are recruited.

In this model, only *in situ* rewarming (i.e. the arousal group), but not *ex vivo* rewarming (i.e. the 7-days torpor group), resulted in the normalization of contractile properties of aortic tissue as compared to non-hibernating animals. This implies that a systemic factor, i.e. a factor not present in or produced by the vascular tissue during rewarming, regulates the contractility during hibernation. A number of studies have suggested the existence of a blood-borne "hibernation inducing trigger", a poorly characterized compound derived from plasma of hibernating animals with delta-opioid agonist characteristics and capable of inducing hibernation or hibernation-like responses in different animal species [26–31]. Furthermore, plasma from hibernating woodchucks or black bear induced an extended preservation of whole lungs [32] and a decrease in ischemia/reperfusion injury of the isolated heart [33,34]. In those hearts, an improved coronary perfusion was reported in the group treated with

plasma from hibernators as compared to non-hibernators [33,34]. Taken together, these experiments suggest that it might be of interest to explore the effects of pretreatment with plasma from hibernating animals, hibernation inducing trigger or delta-opioid agonists on cooling/rewarming of vascular tissue.

In conclusion, this study shows that both contractile and relaxing properties of aortic tissue of hibernating European ground squirrel aorta return to normal within 1.5 h of arousal. Normalization of contractile properties in arousal is accomplished by a rapid offset of the increased sensitivity to vasoconstrictors and accompanied by an increased basal NO production. These adaptive mechanisms are induced by a systemic factor, since they were lacking in rings rewarmed *ex vivo*.Further, the involvement of the NO-pathway in agonist-mediated relaxation differs between the various stages of hibernation. Identification of the mechanisms underlying these changes may be employed to preserve vascular performance and improve organ function after prolonged cold storage.

Limitations

The European ground squirrel does not represent a standard laboratory animal, hence only a limited number of animals per study group were included in this study. Also, the animals do not represent an inbred animal model. Both factors have contributed to a substantial inter-animal variation in response, which limit the power of statistical evaluation. Therefore, more subtle differences in aortic adaptation during arousal may have gone unnoticed.

In addition, resting tension in torpor groups was set at 4 °C, whereas it was set at 21 °C in the other groups. By employing isotonic contraction measurement we attempted to avoid a possible influence. However, it cannot be excluded that this procedure may have led to a systematical error.

To obtain insight into potential alterations in the vascular NO-pathway during various phases of hiberation, we employed a well-defined inhibitor of endothelial NO-synthase. Because of limitations in the availability of material and other reasons, we did not additionally use blockers (for example, indomethacin was not used as it provided a strong suppression of Angiotensin II-mediated contraction) of additional vasoactive pathways (e.g. EDHF, prostanoids) that might be active (both basal as well as after ACh) together with NO. Therefore, we cannot exclude potential interactions among these vasoactive substances nor can we comment on the relevance and the nature of the dilatory response after ACh that was resistant to L-NMMA. Conclusive evidence regarding alterations in vascular NO-pathways will require additional studies, preferably in combination with the assessment of different NOS-isoenzymes and experimental techniques such as determination of NOS protein or mRNA.

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