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Differential regulation of glomerular and interstitial endothelial nitric oxide synthase expression in the kidney of hibernating ground squirrel

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Abstract

Hibernating animals transiently reduce renal function during their hypothermic periods (torpor), while completely restoring it during their periodical rewarming to euthermia (arousal). Moreover, structural integrity of the kidney is preserved throughout the hibernation. Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) is a crucial vasodilatory mediator and a protective factor in the kidney. We investigated renal NOS expression in hibernating European ground squirrels after 1 day and 7 days of torpor (torpor short, TS, and torpor long, TL, respectively), at 1.5 and at 10 h of rewarming (arousal short, AS, and arousal long, AL, respectively), and in continuously euthermic animals after hibernation (EU). For that purpose, we performed NOS activity assay, immunohistochemistry and real-time PCR analysis. Immunohistochemistry revealed a decreased glomerular eNOS expression in hibernating animals (TS, TL, AS, and AL) compared to non-hibernating animals (EU, $p < 0.05$), whereas no difference was found in the expression of interstitial eNOS. Expression of iNOS and nNOS did not differ between all groups. The reduced glomerular eNOS was associated with a significantly lower eNOS mRNA levels and NOS activity of whole kidney during torpor and arousal (TS, TL, AS, and AL) compared to EU. In all methods used, torpid and aroused squirrels did not differ. These results demonstrate differential regulation of eNOS in glomeruli and interstitium of hibernating animals, which is unaffected during arousal. The differential regulation of eNOS may serve to reduce ultrafiltration without jeopardizing tubular structures during hibernation.

Keywords: Hibernation; Kidney; Nitric oxide synthase; *Spermophilus citellus*

Mammalian hibernation has been perceived as a natural model of kidney preservation for clinical conditions such as renal ischemia and transplantation [1]. Particularly small hibernating animals seem suited as a model, because during hibernation they cycle through periods of extremely low body temperature (0–4 °C; torpor phases) and full rewarming (35–37 °C; arousal phases). Besides the nearly complete shutdown of metabolic function, torpor is characterized by dramatic decrease in heart rate [2] and blood pressure [3], increased peripheral resistance and shunting of the blood to the heart, brain, lungs, diaphragm, and brown adipose tissue [4,5].

Consequently, as the kidney is reset to function at minimal blood flow during torpor, renal hemodynamics and function undergo dramatic changes, reflected in a reduction of glomerular filtration rate (GFR) [6–8], and effective renal plasma flow (ERPF) [5,8,9] below 5–10% of their normal values. However, during periodic arousals, cardiac output and body temperature normalize

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rapidly [10], with full restoration of GFR, ERPF, and urine production [6,8]. Although recent studies suggest that animals suffer from oxidative stress during hibernation [11], they maintain (ultra)structural integrity of the kidney [1].

To date, there is only limited information on the molecular basis of the adaptive features employed by hibernators to prevent or counteract kidney damage. Recent studies examining changes in vascular properties of hibernating animals have suggested nitric oxide (NO) to play an important role [12–14]. In the kidney of non-hibernating animals, NO is a key factor involved in the control of renal hemodynamics and function. In various species all three isoforms of nitric oxide synthase (NOS) have been identified in the kidney, i.e. the constitutive forms, endothelial and neuronal NOS (eNOS and nNOS, respectively) and the inducible form (iNOS). Nitric oxide contributes to the modulation of afferent arteriolar tone [15] and mesangial cell contraction [16], thereby modulating the renal hemodynamics and function. Further, studies in different pathophysiological conditions, including renal ischemia–reperfusion, have identified eNOS as a protective NO-producing enzyme [17,18]. Moreover, NO inhibits platelet aggregation and adhesion molecules expression, as well as vascular smooth muscle and mesangial cell proliferation and modifies oxidative tissue damage [19]. Therefore, NO may contribute to the changes in renal hemodynamics during hibernation and/or to the maintenance of the normal renal structure and function during the cyclic ischemia/reperfusion that the kidney undergoes during hibernation.

In this study, we examined the regulation of renal NOS in hibernation by determining its expression in the European ground squirrel (*Spermophilus citellus*) at different time points in torpor and arousal during hibernation, and in continuous euthermia in spring, by the means of NOS activity assay, immunohistochemistry and real-time PCR.

Experimental procedures

Animals

The European ground squirrels (*S. citellus*) used in our study were captured and housed as described previously [12]. Briefly, 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). Rabbit breeding chow and water were provided ad libitum. The animals were housed in a climate-controlled room at a relative humidity of 60%. To induce hibernation, the environmental temperature was gradually reduced from 20 to 5 °C and the light conditions were changed from 12 h light and 12 h dark, to continuous dim red light (<1 lux). To assess the individual torpor-arousal patterns, the nestbox temperatures were measured every minute using a computer based recording system [20]. Furthermore, six squirrels were equipped with a customized abdominal temperature logger (Fidbit, Onset, USA) to register body temperature every 48 min.

Animals were sacrificed at several time points of the hibernation cycle: (1) torpid animals for 1 day (torpor short group, TS; $n = 5$), (2) torpid animals for 7 days (torpor long group, TL; $n = 5$), (3) aroused animals after 1.5 h (arousal short group, AS; $n = 5$), and (4) aroused animals after 10 h (arousal long group, AL; $n = 5$). In addition, material was collected after cessation of hibernation from (5) euthermic animals (EU; $n = 8$). The animals were allowed to hibernate at least 10 weeks before being sacrificed. Average of the spontaneous torpor bout duration was 11.2 ± 0.4 days, and of the spontaneous arousal episode duration was 20.6 ± 0.9 h. At the time of the experiment, the duration of their torpor bouts did not differ between torpor and arousal groups (7.0 ± 0.2 and 7.0 ± 0.1 days, respectively). The euthermic animals were studied 6–7 days after cessation of hibernation in spring. Animals were terminally anesthetized (thiopental, 120 mg, i.p.) and blood was collected by aortic puncture. Renal tissue was removed and snap-frozen in liquid nitrogen and stored at −80 °C. Animal experimental protocols were approved by the Animal Experiments Committee of the University of Groningen, The Netherlands (BG02198).

Blood analysis

Serum creatinine, urea, and electrolytes were measured by an automated multi-analyzer (SMA-C, Technicon Instr Corp., Tarrytown, NY, USA). Combined total nitrite and nitrate concentrations (NO*x*) were determined in plasma using a modified Griess reaction, as previously described [21], in both hibernating ($n = 2$–4/group) and euthermic animals ($n = 8$). The measurements from short and long aroused animals were pooled in one group "arousal" (A) and the measurements from short and long torpid animals were pooled in one group " torpor" (T).

NOS activity assay

Nitric oxide synthase activity was measured by the conversion of radio-labeled L-[^{3}H]arginine to L-[^{3}H]citrulline, using a NOS assay kit (Stratagene Europe, Amsterdam, The Netherlands), according to the manufacturer's protocol. In brief, 5 µl of tissue protein extract from the whole kidney homogenate was added to 40 µl of reaction mixture consisting of 50 mM Tris–HCl, pH 7.4, 6 µM tetrahydrobiopterin (BH4), 2 µM flavin adenine dinucleotide (FAD), 2 µM flavin adenine mononucleotide (FMN), 10 mM NADPH, 6 mM $CaCl_2$ and 1 µCi/µl [^{3}H]arginine. Rat cerebellum extract was used as

a positive control. Duplicate reactions were carried out in the absence and presence of *N*ω-nitro-L-arginine methyl ester (L-NAME, 1 mM), a specific NOS inhibitor. Incubation was performed at 37 °C, for 40 min and the enzyme reaction was stopped by adding 400 μl of stop buffer (50 mM Hepes, pH 5.5 and 5 mM EDTA). L-[^{3}H]Citrulline was then separated from the incubation mixture by addition of an equilibrated resin, followed by centrifugation at 14 000 rpm, for 30 s. The specific NOS activity was determined by subtracting the counts obtained in the presence of L-NAME, from the counts obtained in the absence of L-NAME. Protein concentration of the renal samples was determined using a Bio-Rad protein assay. The results are expressed as pmol of citrulline formed, per mg protein, per 40 min.

eNOS, nNOS, and iNOS immunohistochemistry

Cryostat sections were cut at 4 μm and fixed in acetone for 10 min. Sections were then treated with 0.075% H_2O_2 in PBS, pH 7.4, for 30 min, to block the endogenous peroxidase (PO). eNOS, nNOS, and iNOS proteins were detected using monoclonal mouse antibodies (Transduction Laboratories, Lexington, KY, USA), as described previously [21]. A two-step immunoperoxidase technique was performed, with sequential incubations of PO-labeled rabbit anti-mouse and PO-labeled goat anti-rabbit (all from Dakopatts, Glostrup, Denmark). All PO-labeled antibody dilutions were made in PBS, pH 7.4, supplemented with 1% normal squirrel serum. Peroxidase activity was developed using a freshly prepared solution of 3-amino-9-ethylcarbimazole and H_2O_2. Sections were counterstained with hematoxylin. Negative controls were performed by replacing the primary antibodies either by PBS or by nonspecific antibodies of the same IgG isotype. Control sections were consistently negative (data not shown). The intensity of immunostaining was scored semiquantitatively by an observer blinded for the groups, from 0 to 3, as follows: 0 = absent; 1 = weak; 2 = moderate; and 3 = strong.

RNA isolation

Frozen kidneys tissue was brought into tubes containing 2 ml of lysis buffer (GIT) consisting of 4 M guanidine thiocyanate, 25 mM Na citrate, 0.5% *N*-lauroyalsarcosine and 10 UI/ml β-mercaptoethanol added just before use. The tissue was homogenized on ice, then 0.2 ml 2 M Na acetate and 2 ml water-saturated phenol were added. Total RNA was then extracted with 0.4 ml chloroform, followed by 10 min centrifugation at 8500 rpm at 4 °C. Supernatant was transferred to a new tube and the RNA was precipitated with isopropanol (vol/vol). After another 10 min centrifugation at 12 000 rpm at 4 °C, the supernatant was removed and the pellet was resuspended into 0.5 ml GIT and 1 vol isopropanol. The mixture was pelleted again by centrifugation at 14 000 rpm at 4 °C, for 10 min, washed with 0.5 ml 75% ethanol, air-dried and reconstituted in diethyl pyrocarbonate-treated water. Integrity of RNA was determined using agarose gel electrophoresis and the RNA concentration was measured by spectrophotometry at 260 nm.

Real-time PCR

The expression of eNOS in squirrel kidney was analyzed using real-time two-step quantitative RT-PCR. Quantification was performed with SYBR Green PCR reagents (Molecular Probes Europe, Leiden, Netherlands) and an ABI PRISM 5700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, Netherlands). A 50 μl PCR mixture contained 0.5 unit *Taq* polymerase (Eurogentec, Belgium), 5 μl of the supplied reaction buffer, 250 nM dATP, 250 nM dCTP, 250 nM dGTP, 500 nM dUTP, 2 mM $MgCl_2$, 50 ng cDNA, 500 nM of each gene specific primer, 1 μl of 50× ROX reference dye (Invitrogen, Breda, Netherlands) and 1 μl of 10∗Sybr Green I (Molecular Probes Europe, Leiden, Netherlands). The PCR profile consisted of 5 min at 95 °C, followed by 40 cycles with heating to 95 °C for 15 s and cooling to 60 °C for 1 min. PCR product specificity and purity was evaluated by gel-electrophoresis and by generating a dissociation curve following the manufacturer's recommendations. Sample CT values were normalized to CT values for 18S RNA. Sequence-specific PCR primers were purchased from Eurogentec (Seraing, Belgium). The PCR primers used were as follows. eNOS: sense, 5′-GG CATCACCAGGAAGAAGACC-3′; antisense, 5′-GCC ATCACCGTGCCCAT-3′. 18S: sense, 5′-CATTCGAAC GTCTGCCCTATC-3′; antisense, 5′-CCTGCTGCCTT CCTTGGA-3′. To confirm amplification of eNOS in the PCR, the amplified product was sequenced (GenBank Accession No.: AY177690). The product showed a 100% homology with eNOS from mouse.

Statistical analyses

Values are expressed as means ± SEM, unless stated otherwise. Differences between groups were analyzed using one-way ANOVA with Student–Newman–Keuls correction for pairwise comparisons for continuous variables, and Kruskal–Wallis ANOVA on ranks followed by Dunn's test for discreet variables. Differences were considered significant at $p < 0.05$.

Results

Timing and body temperature

Daily nest box temperature registration confirmed the presence of torpor/arousal patterns in hibernating animals

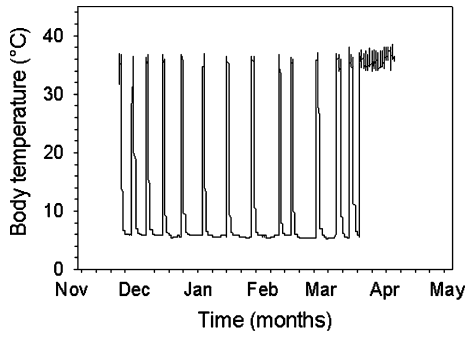

Fig. 1. Typical registration of the abdominal temperature by an implanted abdominal temperature logger, demonstrating the torpor/arousal pattern during hibernation under laboratory conditions in the European ground squirrel.

under laboratory conditions (Fig. 1). Measurement of rectal temperature at the moment of removal of the kidney established that the groups represented the targeted phases of hibernation (AS: 30.9 ± 3.6 °C, AL: 34.5 ± 0.7 °C, TS: 8.2 ± 0.5 °C, TL: 8.2 ± 0.6 °C, and EU: 36.5 ± 1.3 °C, respectively).

Blood analysis

To obtain information about renal function during hibernation, serum urea, creatinine, and electrolytes were measured (Fig. 2). Urea did not significantly change during hibernation, while creatinine showed increased values in TL and AS, which normalized at AL and were maintained low at TS. Further, serum phosphate and Mg^{2+} showed a similar pattern of normalization of levels after arousal at AL, with increased levels in TS and particularly in TL. Hibernating animals normalized serum levels of all changed parameters by the end of arousal, except for K^+, which was decreased throughout hibernation. Measurements in urine failed, as the bladders of aroused and euthermic animals were empty at the time of sacrifice. However, previous studies showed that the parameters measured in plasma correlate with the values

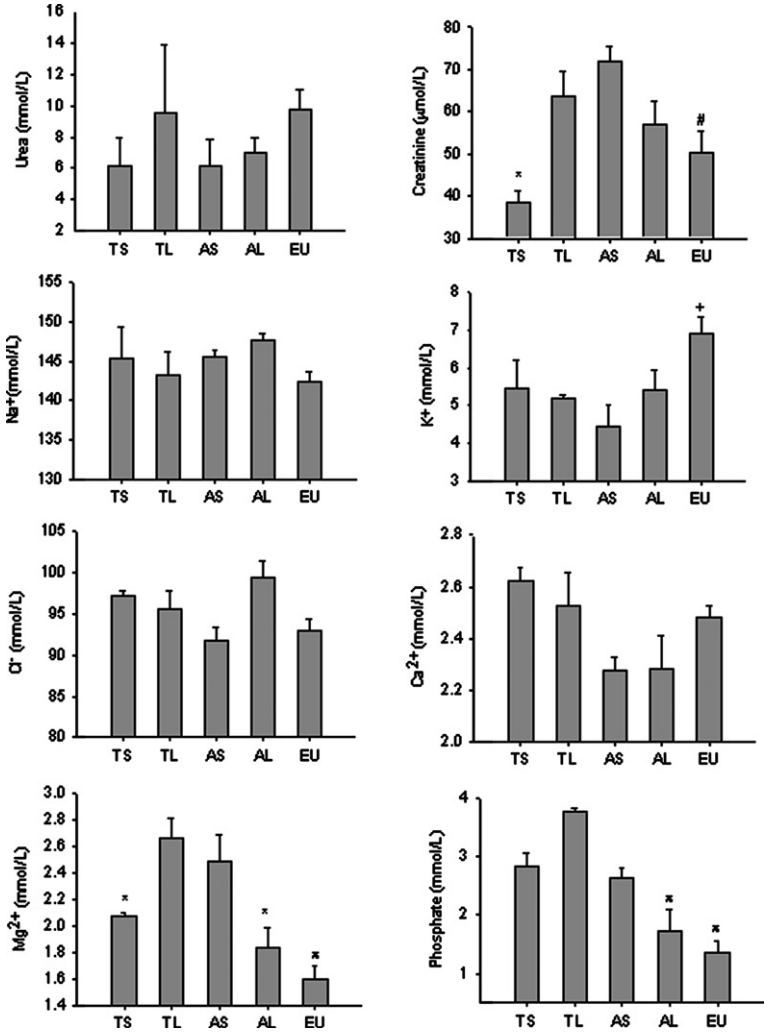

Fig. 2. Changes in serum levels of urea, creatinine and electrolytes related to the arousal period in hibernation (all groups $n = 5$, except EU $n = 8$). (∗) significantly different compared to TL, AS; (#) compared to AS; (+) compared to all hibernating groups, and (π) compared to TS, TL, and AS.

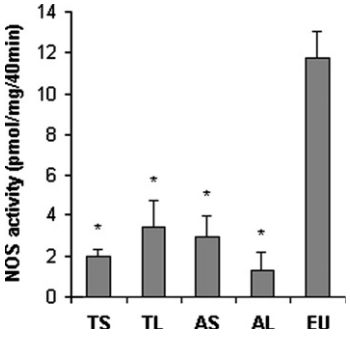

Fig. 3. NOS activity levels during hibernation: NOS activity was reduced in both torpid (TS and TL) and aroused (AS and AL) animals compared to euthermic (EU) animals.

measured in urine both during hibernation and euthermia [6,22].

Plasma NO*x* levels were significantly lower both during torpor (T: 56.25 ± 9.88 μmol/L) and arousal (A: 51.14 ± 10.75 μmol/L) compared to euthermia (EU: 151.8 ± 45.10 μmol/L) ($p < 0.05$).

NOS activity

The results of in vitro NOS activity assay are presented in Fig. 3. A significantly lower level ($p < 0.01$) of NOS activity was found during both torpor (TS: 2 ± 0.32 pmol/mg/ 40 min; TL: 3.43 ± 1.27 pmol/mg/40 min) and arousal (AS: 2.95 ± 1.01 pmol/mg/40 min; AL: 1.32 ± 0.85 pmol/mg/40 min) when compared to euthermic animals (EU: 11.73 ± 1.33 pmol/mg/40 min). No significant difference ($p > 0.05$) was found between the hibernating groups (TS, TL, AS, and AL).

eNOS, nNOS, and iNOS immunostaining

In view of the reduced overall NOS activity found during hibernation, we determined the expression of all three NOS isoforms protein in the squirrel kidney by immunohistochemical staining. In euthermic animals, expression of eNOS was found in glomeruli, and within the in interstitium in peritubular capillaries and arterioles. No eNOS staining was found in tubules. A strongly reduced eNOS immunoreactivity was found in the glomeruli of both torpor ($p < 0.01$) and arousal ($p < 0.05$) groups compared to euthermic animals (Fig. 4). In contrast, eNOS expression at the interstitial level did not differ between the groups ($p > 0.05$). There was no difference between the hibernating animals (TS, TL, AS, and AL) either in glomerular or interstitial eNOS expression.

nNOS protein was localized mainly in the macula densa in the cortex, but also in the medulla, while expression of iNOS protein was found in the tubular structures of both cortex and medulla. No significant changes could be detected in the staining intensity of both nNOS and iNOS between the groups ($p > 0.05$, data not shown).

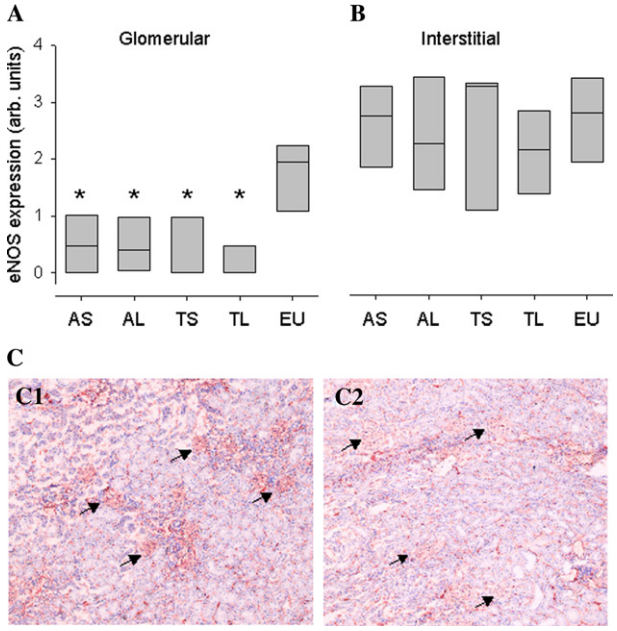

Fig. 4. Immunostaining for eNOS in squirrel kidneys. (A) Reduction of glomerular eNOS expression in torpor groups (TS, TL) and arousal groups (AS, AL) compared to euthermic group (EU). Torpid and aroused animals did not significantly differ in glomerular eNOS expression ($p > 0.05$). (B) No significant change in the interstitial eNOS expression was found between the five groups of squirrels. Horizontal line denotes median, boundary of the box indicates the 25th percentile and 75th percentile and bars indicate 10th and 90th percentiles. (C) Representative pictures from immunohistochemistry: decreased glomerular (arrows) eNOS expression can be seen in a hibernating squirrel (arousal) (C1), compared to glomeruli from a euthermic squirrel (C2). *$p < 0.05$ compared to euthermic animals.

Real-time PCR

To further investigate the mechanism of reduced eNOS expression during hibernation, eNOS mRNA was measured by real-time RT-PCR on whole kidney homogenates and expressed as ratio to 18S mRNA (Fig. 5). The mRNA levels of eNOS were significantly reduced during torpor (TS: 49.41 ± 9.52, TL: 43.83 ± 20.04, $p < 0.05$), compared to euthermia (EU: 112.64 ± 18.06). There was a trend towards increased eNOS mRNA

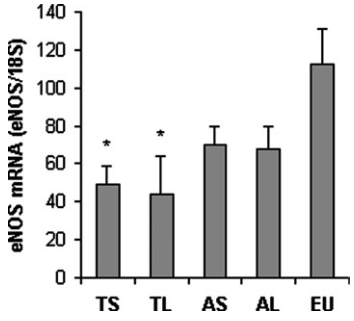

Fig. 5. eNOS mRNA levels during hibernation: eNOS mRNA was significantly reduced in torpid animals (TS, TL) compared with euthermic animals (EU). (*) Significantly different compared to EU.

levels during arousal (AS: 69.97 ± 9.82, AL: 67.65 ± 12.08) compared with torpor, though the values were at the border of statistical significance ($p = 0.06$).

Discussion

The present study examines the changes in the renal expression of NOS in the European ground squirrel during hibernation. The major finding is a significant decrease in glomerular eNOS expression throughout hibernation, without a change in interstitial expression. In contrast, expression of nNOS and iNOS was unaffected by hibernation. The decrease in glomerular eNOS expression during torpor was associated with decreases both in whole kidney eNOS mRNA levels and NOS activity. Furthermore, arousal did not affect the expression of eNOS or NOS activity levels when compared to torpor. Thus, our data suggest that ground squirrels prepare their kidney for hibernation by a specific downregulation of glomerular eNOS through a decrease of its gene transcription.

It has previously been shown that, during hibernation, there is a reduction of glomerular filtration rate (GFR) to less than 10% of that found in a euthermic animal [8]. At a single nephron level, GFR is the result of two major factors: the filtration pressure across the glomerular capillary, and the ultrafiltration coefficient. Endothelial-derived NO is known to modulate GFR, by influencing both these factors. First, NO is an endogenous vasodilator, acting mainly on the preglomerular arterioles [23]. Thus, a reduced NO production in the hibernating kidney might decrease the net filtration pressure. Second, NO has been shown to inhibit contraction of the mesangial cells [16]. Hence, a reduced eNOS expression decreases the glomerular filtration surface. Considering both, these would result in a reduced GFR. Such a reduction of NO-mediated renal vasodilatation might allow endogenous vasoconstrictors, e.g., angiotensin II, to predominate and mediate an increased vascular resistance within the kidney. Indeed, the renin–angiotensin system seems to be activated during hibernation, since plasma renin and juxtaglomerular cells activity are increased in this period, compared with euthermia [24,25]. Thus, downregulation of glomerular eNOS may principally serve to reduce GFR during hibernation.

Previous studies have indicated that GFR, ERPF and tubular excretion are fully restored during arousal periods [6,8]. In agreement, we found that the increased serum levels of creatinine, $Mg^{2+}$and phosphates at late torpor are normalized during the arousal period. Interestingly, serum levels of Mg^{2+} and phosphates normalized faster than those of creatinine, which may reflect the observation that the restoration of tubular activity is an earlier event than normalization of GFR in aroused hibernators [9]. In this regard, the preserved eNOS expression at the peritubular capillaries may serve to secure adequate blood supply to the tubular cells throughout hibernation enabling the animal to immediately restore tubular function during arousal.

Normalization of glomerular filtration during arousal seems largely independent of eNOS, as we did not find any significant change in its expression and NOS activity during the arousal phase, when compared to torpor. Restoration of renal vascular function during arousal periods may therefore depend on vasorelaxing pathways other than NO, as was found in the aorta of aroused ground squirrels [12].

While our data demonstrate a regional control of eNOS expression within the kidney, it may even be more complex in the whole hibernating animal. Previously, the expression of eNOS protein in the hamster renal artery was reported to be downregulated during torpor and upregulated during arousal [13]. We did not measure the eNOS protein expression in the renal artery in our study, however we did not find changes in eNOS expression either in the renal arterioles or peritubular capillaries. Whether this reflects differences between vascular beds or species remains to be established.

The most straightforward interpretation of our data is that downregulation of eNOS mRNA drives the decrease in glomerular eNOS and consequently NOS activity is reduced, however several other factors may well take part therein. The decrease in glomerular eNOS protein expression seems transcriptionally regulated, since we found an approximately 2-fold decrease in eNOS mRNA levels during hibernation. As regulation of glomerular eNOS appears independent of the specific phase of hibernation, it seems conceivable that its expression is driven by factor(s) related to the hibernating state as such, rather than in response to changes at the renal level. Surprisingly, the glomerular eNOS protein reduction was associated with 4-fold decrease in overall NOS activity. As it seems unlikely that the reduction in expression of glomerular eNOS can account for such a strong reduction of total NOS activity we studied also the expression of the other two NOS isoforms. Yet, no measurable changes in either nNOS or iNOS protein expression were found. Consequently, additional factors are likely to be involved in reducing renal NOS activity during hibernation. However, by our method we did not assess the possible changes in co-factor or substrate availability or the presence of NOS modulators (such as ADMA [26], caveolin-1 [27], etc.) that might well affect NOS activity in vivo. A potentially interesting finding is that NO*x* plasma levels, which reflect the NO production in all tissues, showed the same pattern as NOS activity in the kidney. In which other organs than kidney the NO system is regulated during hibernation remains to be established.

While decrease in glomerular eNOS may be advantageous for renal hemodynamics during hibernation,

reduced eNOS expression has been associated with extensive renal damage in pathological conditions because of detrimental effects on neutrophil infiltration and platelet aggregation [28]. Also, increased NO production by L-arginine supplementation attenuates renal ischemia–reperfusion injury in rat [29]. Since we found a reduced glomerular eNOS expression throughout hibernation, other physiological mechanisms operating in hibernation, such as sequestration of leukocytes and increase in blood clotting time [30], may be involved here in obtaining renal tolerance to ischemia.

In conclusion, throughout hibernation there is a downregulation of eNOS expression in the glomerular capillaries, without a change in expression in the interstitial vasculature, which is unaffected during the arousal period. Whether such regional regulation of eNOS provides a feasible strategy to preserve renal function in a clinical situation awaits clarification of its mechanism.

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