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Role of renal nerves and salt intake on erythropoietin secretion in rats following carbon monoxide exposure*

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Abbreviations:

CO, carbon monoxide; EPO, erythropoietin; DNX, bilateral renal denervation; INN, intact renal innervation; GFR, glomerular filtration rate; ISO, isoproteronol; PROP, propranolol; NPY, neuropeptide Y; ANPY, specific NPY antagonist; PRA, plasma renin activity; AII, angiotensin II; LS, low salt diet; HS, high salt diet; NS, normal salt diet

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Abstract

Since the data from the literature contain conflicting results about the role of renal nerves and angiotensin II in hypoxia-induced erythropoietin (EPO) secretion we evaluated the effect of renal nerves and salt intake in rats on EPO secretion stimulated by carbon monoxide (CO).

Serum levels and renal mRNA content of EPO were similarly elevated by exposure to different CO concentrations in a dose dependent manner in rats with bilateral renal denervation (DNX) and in sham-denervated controls (INN). However, at 600 ppm CO, serum concentrations and mRNA of EPO were significantly higher in DNX compared to INN rats ($p<0.05$). This increase of EPO secretion in DNX rats could be blocked by administration of neuropeptide Y (NPY) ($p<0.05$), whereas the NPY receptor antagonist (NPYA) did not enhance EPO secretion in INN rats following CO exposure. Agonists and antagonists of beta-adrenergic receptors had no effect on EPO secretion. High salt diet (HS) reduced EPO secretory response at 600 ppm CO by 55% compared to INN rats on normal salt diet ($p<0.01$). DNX increased EPO secretion also in rats on LS and HS diet whereas plasma renin activity did not correlate with EPO levels under these experimental conditions. In summary, our data suggest that renal nerves contribute to the half-maximal EPO secretory response to CO exposure, possibly via NPY receptors.

Introduction

Erythropoietin (EPO), a glycoprotein hormone, is an essential growth factor which regulates erythropoiesis and is, in adult laboratory animals and in humans, predominantly synthesized in the kidneys (Jelkmann, 1992; Jelkmann and Metzen, 1996). EPO secretion and renal mRNA content are inversely correlated to renal oxygen supply (Scholz, et al., 1991). Since renal ischemia in isolated perfused kidneys elicited a much weaker EPO secretory response compared to systemic hypoxia in vivo, it was postulated that additional factors like nerves and humoral factors may contribute to EPO secretory response to hypoxia (Pagel, et al., 1989).

Despite of the fact, that multiple studies have investigated the role of renal nerves on EPO secretion, the published data are contradictory. Beynon et al. reported increased EPO levels in rats exposed to 6 h of hypoxia (10% O_2) following denervation (DNX) of the left kidney and after contralateral nephrectomy compared to control rats with intact renal nerves (Beynon, 1977). In contrast Fink and Fisher found reduced EPO levels in rabbits exposed to 5 hours of hypoxia (0.42 atm) following bilateral renal denervation in control animals (Fink and Fisher, 1976). Eckardt et al. showed in rats that denervation of one kidney does not effect EPO mRNA following systemic exposure to hypoxia, haemorrhage and carbon monoxide (CO) compared to the contralateral innervated control kidney (Eckardt, et al., 1992).

Moreover, combination of renal denervation and beta-adrenergic receptor blockade showed to inhibit the hypoxia-stimulated EPO secretion compared to controls (Fink and Fisher, 1976). However, Jelkmann et al. could not confirm these findings since his group reported unchanged EPO levels following long-term administration of a selective beta-2 adrenergic agonist in rabbits (Jelkmann, et al., 1979). Studies in humans showed that EPO secretion was higher following administration of fenoterol, a beta2-adrenergic receptor agonist following haemorrhage and under normoxic conditions (Gleiter, et al., 1997;

Freudenthaler, et al., 1999a; Gleiter, et al., 1998). Despite of these efforts, the role of renal nerves on EPO secretion remains only poorly understood.

Neuropeptide Y (NPY), another co-transmitter of the sympathetic nervous system, is discussed to be involved in regulation of EPO secretion. NPY is present in almost all renal sympathetic nerve endings (Chevendra and Weaver, 1992) and was shown to be absent in kidneys following renal denervation (Chevendra and Weaver, 1992; Eckardt, et al., 1992). Furthermore NPY is involved in renal hemodynamics by inducing vasoconstriction with only little effect on glomerular filtration rate (Bischoff and Michel, 1998).

Another systemic factor that could modulate renal EPO secretion is the renin-angiotensin system. Infusion of angiotensin II (AII) into the renal artery of dogs and an increase of endogenous AII following reduction of renal perfusion pressure increased EPO serum levels (Fisher, et al., 1967). Studies in human volunteers demonstrated that elevation of plasma renin activity (PRA) and thus AII levels as well as intravenous infusion of AII elevated in a dose dependent fashion EPO concentrations. These effects were greatly reduced by administration of the AII receptor antagonist losartan (Gossmann, et al., 2001; Freudenthaler, et al., 1999a; Freudenthaler, et al., 2000). However, alteration of endogenous angiotensin II levels following high and low salt diet showed no effect on EPO secretion in healthy volunteers (Freudenthaler, et al., 2003). Whereas high salt diet increased EPO concentrations in essential hypertensive patients (Naomi, et al., 1993).

Since the current data about the role of renal nerves on EPO secretion are equivocal and since the impact of renal nerves on EPO secretion has never been investigated systematically we re-assessed the role of renal nerves on EPO secretion by constructing a dose response relationship between the degree of hypoxia and EPO secretion. Furthermore, we investigated the effect of agonists and antagonists of beta adrenergic and NPY receptors

on EPO secretion. To alter endogenous AII levels we chose high and low salt diet for three weeks as a long-term modulator of inhibition and stimulation of PRA, respectively of EPO.

Methods

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 250 to 320 g were kept on a regular 12-hour dark-light cycle with free access to standard rat chow (Altromin 1320, Altromin, Lage, Germany) or kept on high salt diet (4.0% NaCl in chow plus 1% NaCl in drinking water) or on low salt diet (administration of furosemid 5mg/kg i.p. and subsequent feeding with 0.015% sodium chow) for three weeks. The rat experiments were conducted in accordance with the German Animal Protection Law.

Experimental design and groups: Seven days following bilateral renal denervation (DNX) rats were exposed to different CO concentrations. Innervated kidneys (INN) in sham-denervated rats served as controls. For constructing a dose-response relationship in DNX and INN rats we used for each CO concentration (0-1000 ppm) 5 up to 8 rats. To compensate the lack of transmitter release in DNX rats we administered [Leu^{31}, Pro^{34}]-Neuropeptide Y (NPY, 0.3 μg/min/kg) or isoproteronol (ISO, 3 μg/h/kg) i.v. via osmotic minipumps implanted 12 hrs before exposure to 600 ppm CO. To block these neurotransmitters in control rats we administered in INN rats the beta-adrenergic receptor blocker propranolol (PROP, 4 mg/kg, i.p. 1 h before CO exposure) or NPY_1 receptor blocker (ANPY, 0.5 μg/min/kg, i.v. via osmotic minipump implanted 12 hrs before CO exposure). To change the endogenous angiotensin II concentration in the plasma we studied the effect of DNX on EPO serum concentrations in rats on high (HS+DNX) and low salt diet (LS+DNX) compared to controls without DNX (HS, LS) before exposure to 600 ppm CO. Rats were sacrificed after 4 hours of CO exposure for taking blood samples and collection of both kidneys being stored at -80°C until analysis.

Renal bilateral denervation: Bilateral renal denervation (DNX) was carried out in anesthetized rats with 70 mg/kg BW ketamine i.p. (Parke-Davis, Freiburg, Germany) and 15

mg/kg BW xylazine i.p. (Bayer, Leverkusen, Germany) as described previously (Muhlbauer, et al., 1997). In brief, following abdominal midline incision the renal arteries and veins were isolated and freed of connective tissue. After cutting all visible nerves the vessels were painted for 2 min with a 10% phenolic alcohol solution. Sham denervation was performed under identical conditions of anesthesia with exposure of the left and right renal hilus for 2-4 min without destruction of renal nerves. Completeness of DNX was assessed by measuring renal norepinephrine content with HPLC at the end of CO exposure and was assumed to be achieved when the norepinephrine content was less than 15% of 200 ng/ml in innervated kidneys of control rats.

Implantation of osmotic minipumps. Rats were anaesthetized with diethyl ether. A lateral 1.5 cm neck incision was made and the subcutaneous tissue was spread to form a pocket on the animal's back and an Alzet osmotic minipump (1003D, Durect Corporation, Cupertino, CA) preloaded with the appropriate solution, was inserted. A polythene catheter fitted to the drug delivery portal of the osmotic minipump was inserted into the external jugular vein. Wounds were closed with non-reabsorptive material (Dermafil 2-0; Dr. Ruhland, Neustadt, Germany).

Cabonmonoxide (CO) experiments. CO was mixed with room air (inflow between 6-10 l/min) to generate CO concentrations between 200 – 1000 ppm CO in the cage. The actual CO concentration was monitored by a CO sensor (Testo, Reutlingen, Germany). Rats were exsanguinated under ether narcosis following 4 hours of CO exposure, blood was collected into serum tubes for determination of hematocrit, PRA and EPO concentrations. Kidneys were freed from connective tissue, removed and frozen immediately in liquid nitrogen by a freeze-clamp technique and stored at -80°C until measurement of catecholamines.

Analytics. Serum EPO concentrations were determined by ELISA with a commercial available Kit (Medac, Germany). Arterial blood samples were analyzed for hematocrit. PRA was determined by its capacity to generate angiotensin I with addition of renin substrate by radio-immunoassay, as described previously (Albinus, et al., 1998).

HPLC (high pressure liquid chromatography). Correct renal denervation was assessed by measuring the renal tissue content of catecholamines using a HPLC method as previously described (Muhlbauer, et al., 1997).

For measurement of HbCO venous blood samples were stored at 0 °C in an airtight closed hematokrit tube until measurement by spectophotometry within 30 minutes after collection.

Real Time PCR. Total RNA from rat kidney was isolated using peqGold RNApure (PeqLab, Erlangen, Germany). Synthesis of cDNA was performed using oligo dT_{15} and random hexamers as primers and avian myeloblastosis virus reverse transcriptase (PeqLab, Erlangen, Germany). PCR was carried out on the Lightcycler instrument with the FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany). Primer sequences used for amplification were as follows:

EPO (RefSeq Acc. No. NM_017001): sense: 5'-^{1272}CACGAAGCCATGAAGACAGA^{1291}-3', antisense: 5'-^{1372}GGCTGTTGCCAGTGGTATTT^{1353}-3';

PPIA (NM_017101): sense: 5'- ^{166}GGGGAGAAAGGATTTGGCTA^{185}-3', antisense: 5'-^{422}ACATGCTTGCCATCCAGCC^{404}-3'. Peptidyl prolyl isomerase A (PPIA, cyclophilin A) served as internal control. Standard samples were included for comparison between different PCR runs. The relative expression ratio of the target gene EPO was calculated according to Pfaffl, 2001 (Pfaffl, 2001).

In situ hybridization. Kidneys of control rats (INN) obtained after 4 hours CO-exposure to 400 and 1000 ppm were fixed in 4% paraformaldehyde /0.1 M sodium phosphate buffer (pH 7.2) over night and embedded in paraffin. Five µm tissue sections were dewaxed and hybridized as previously described (Klingel, et al., 1992). Hybridization probes at a length of 502 bp were generated by in vitro transcription of a mEPO cDNA cloned into pGEM-3Z SP6 using T7 polymerase (antisense). The hybridization mixture (10 mM Tris HCl, pH 7.4, 50% (vol/vol) deionized formamide, 600 mM NaCl, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% bovine serum albumin, 10% dextrane sulfate, 10 mM dithiothreitol, 200 µg/ml denatured sonicated salmon sperm DNA, 100 µg/ml rabbit liver tRNA) contained either the ^{35}S-labeled RNA antisense EPO or an unrelated plasmid vector control RNA probe at a concentration of 500 ng/ml. Hybridization with RNA probes proceeded at 42ºC for 18 hr. Following washing steps, the slide preparations were dipped in NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4°C for 3 weeks. After development the slides were stained with hematoxylin/eosin.

Chemicals. All chemicals, the neuropeptide Y agonist [Leu^{31},Pro^{34}]-neuropeptide Y (NPY), the neuropeptide Y_1 receptor antagonist Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic (2,4`)(2`,4) diamide (ANPY), propranolol (PROP) and isoproteronol (ISO) were purchased from Sigma-Aldrich, Steinheim, Germany.

Statistical methods. Data between the different groups were compared by the unpaired Student`s *t* test, or by analysis of variance (ANOVA). All values are presented as mean ± SEM. $p<0.05$ was considered to be statistically significant.

Results

Degree of hypoxia: In order to characterize our model of systemic deficiency of oxygen delivery we measured CO binding to haemoglobin in rats exposed to increasing CO concentrations in air (n = 5-8 per group). As shown in Fig. 1 increased concentrations of CO in inspired air for four hours leads to an increase of HbCO up to 56 % following exposure to 1000 ppm CO. The increased HbCO concentration enhances sharply the O_2 binding affinity of haemoglobin and thus reduces the O_2 delivery to the tissue (Coburn and Forman, 1987). Our in vivo data are in good aggreement to those of Coburn et al. (Coburn and Forman, 1987). Hematocrit values were between 44 and 48 % following CO exposure in all groups.

In order to localize the cell types in kidneys which express EPO mRNA following exposure to different CO concentrations we performed *in situ* hybridization experiments. As shown in Fig. 2, EPO mRNA is detected exclusively in cells of the peritubular space mainly located in the deep cortical juxtamedullary region. The density of silver grains indicating EPO mRNA of the interstitial kidney cells was much higher following severe hypoxia (1000 ppm, Fig. 2A) compared to those kidney cells from rats following exposure to 400 ppm (Fig. 2B). No autoradiographic signals were detected in tissue specimens of the kidneys when hybridized with the ^{35}S-labeled plasmid control RNA probe (data not shown).

Effect of renal nerves: EPO serum concentrations were significantly elevated following CO exposure (0-1000 ppm) in a dose dependent manner in both INN and DNX rats resulting in a 4-, 7-, 96- and 190-fold increase following exposure to 200, 400, 800 and 1000 ppm CO, respectively (Fig. 3A). Renal EPO mRNA increased significantly in parallel to EPO serum levels following exposure to different CO concentrations compared to baseline concentrations without a difference between rats with or without renal denervation (Fig. 3B). EPO serum levels in DNX rats, however, were two fold higher following exposure to 600 ppm (527 ± 48 mU/ml) compared to INN rats (250 ± 41 mU/ml, Fig. 3A, $p<0.05$), indicating

a higher sensitivity of DNX rats to CO which stimulates EPO secretion half maximal. The increase of EPO serum concentrations at 600 ppm in rats with renal denervation was accompanied by a significant higher renal mRNA content in these rats compared to the respective INN rats ($p<0.05$, Fig. 3B).

This increase of EPO serum concentrations in DNX rats following exposure to 600 ppm CO could be blocked by administration of the neuropeptide Y agonist [Leu^{31},Pro^{34}]-neuropeptide resulting in similar EPO serum concentrations compared to INN rats ($p<0.05$, Fig. 4A). EPO mRNA content was decreased in parallel to EPO serum concentrations ($p<0.05$, Fig. 4B). However, the administration of the selective NPY_1 receptor antagonist Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic (2,4`)(2`,4) diamide in INN rats increased only slightly EPO serum levels as well as EPO mRNA but this increase did not reach significance (Fig. 4A/B). Application of isoproteronol in DNX rats as well as propranolol in INN rats showed an insignificant tendency to decrease or increase EPO serum concentrations, respectively (Fig. 4A). Isoproteronol in DNX rats as well as propranolol in INN rats did not modify renal EPO mRNA content (Fig. 4B).

Effect of salt diet on erythropoietin secretion. High salt diet (HS) reduced EPO secretory response by 55% at 600 ppm CO compared to rats on normal salt diet (NS, $p<0.01$, Fig. 5A). However, this inhibitory action of HS was greatly attenuated by DNX with three-fold higher EPO serum levels compared to CO response in INN-HS rats ($p<0.05$, Fig. 5A). This indicates, that HS-induced depression of EPO secretion depends at least in part on intact innervation. This significant difference in EPO secretion capacity was also seen in renal mRNA content (Fig. 5B). Low salt diet (LS) did not change significantly the stimulatory effect of DNX to increase EPO serum concentrations and renal mRNA at 600 ppm CO (Fig. 5A).

PRA following 3 weeks of HS (3.3 ± 1.2 ngAngI/ml/h) was significantly decreased compared to rats on NS (19.2 ± 1.6). PRA showed a 5.6-fold increase (107.7 ± 6.8 ngAngI/ml/h) in rats following 3 weeks of LS. These changes in PRA were not influenced by DNX (HS: 2.7 ±0.9, NS: 20.5 ± 1.3 and LS: 127.8 ± 22.5 ngAngI/ml/h, respectively).

Discussion

Despite the fact that several studies investigated the role of renal sympathetic nerves on the regulation of EPO secretion in the kidney, contradictory results have been reported. Thus, we re-examined the role of renal nerves in CO-induced EPO secretion by constructing a dose-response relationship between EPO serum levels and CO concentrations in inhaled air in conscious rats with innervated (INN) and bilateral denervated kidneys (DNX). Renal denervation was carried out by a standard procedure of combined mechanical dissection and chemical destruction of renal nerves along the renal artery and renal hilus (Muhlbauer, et al., 1997). In contrast to the other studies we determined completeness of renal denervation by measuring renal catecholamine content by a sensitive HPLC method (Muhlbauer, et al., 1997). Furthermore, the bilateral denervation allows a direct comparison of changes in EPO serum concentrations and renal EPO mRNA content following denervation compared to controls. Exposure of rats to hypoxia was performed seven days after denervation. In our model of CO-induced systemic hypoxia we concentrated on the changes of EPO plasma levels. However, it should be mentioned that CO by itself can elicit specific effects on signaling in cells besides its hypoxia dependent stimulation of EPO secretion. An extensive review on the action of CO in many tissues has been published by Wu and Wang (Wu and Wang, 2005). Most recently, several studies reported on beneficial effects of low dose CO (250 ppm) in pathophysiological conditions such as postoperative ileus, endotoxic shock and resuscitation of hemorrhagic shock (Moore, et al., 2005; Mazzola, et al., 2005; Zuckerbraun, et al., 2005). Our analysis of the dose dependent effects of CO on EPO secretion did not reveal an effect of renal innervation at low (200-400 ppm) or high (800-1000 ppm) CO concentrations. If CO can contribute to cell signalling, this effect is likely to be saturated and overridden by the severe reduction of oxygen delivery to the tissue, at least when EPO secretion is considered.

The dose-dependent increase of EPO serum concentrations and renal EPO mRNA following exposure to different CO concentrations was similar in INN and DNX rats. However, DNX rats revealed a significant higher EPO secretory response following exposure to 600 ppm compared to INN rats. In previous studies animals were exposed to only severe and nearly *maximal* hypoxic stimuli (1000 ppm, 10% O_2 or 0.42 atm over 4, 6 or 5 hours, respectively) (Eckardt, et al., 1992; Beynon, 1977; Fink and Fisher, 1976). Therefore, an effect of renal nerves on EPO secretion at *half-maximal* stimulation could not be detected in these experiments. The increase of EPO serum concentrations as well as EPO mRNA following exposure to 1000 ppm CO in our study are in good agreement with the increases reported by Eckardt et al (Eckardt, et al., 1992). The lack of an effect of renal denervation following exposure to 1000 ppm further suggests that this severe hypoxic stimulus with HbCO concentrations of 56% caused a maximum of EPO secretory response independent of renal innervation and plasma renin activity. Furthermore, increased circulating catecholamines during severe hypoxia could mask modulations of EPO production by renal nerves, as suggested previously by Fink et al. (Fink, et al., 1975). The difference of EPO secretory response following 600 ppm CO exposure could be the result of different changes in hemodynamic and tubular transport characteristics between INN and DNX. It is conceivable that denervated kidneys do not respond to centralization of circulating blood with renal vasoconstriction and subsequent fall in GFR to the same extent as innervated kidneys. Besides catecholamines also NPY is a potent renal vasoconstrictor (Bischoff and Michel, 1998). Since a higher GFR imposes to the tubular system a higher work load for electrolyte reabsorption, renal interstitial oxygen partial pressure may be even lower in DNX compared to INN at 600 ppm CO and thus stimulate EPO secretion further. To address this question in future experiments it would be necessary to carry out experiments in anesthetized rats to measure single nephron glomerular filtration rate and local tissue oxygen partial pressure, especially in the outer medullary region where most of the EPO-secreting cells are located.

Another possibility for renal nerves to modulate EPO secretion at 600 ppm CO could be the afferent nerve traffic originating from the kidney following ischemia (Recordati, et al., 1981; Bischoff and Michel, 1998). Since it has been proposed that the central nervous system is involved in the regulation of EPO secretion (von Wussow, et al., 2005) the interrupted input of afferent renal nerve discharge for seven days may have discrete effects on CNS dependent control of EPO secretion.

To address the role of NPY, present in renal nerve endings (Eckardt, et al., 1992), we examined the action of an agonist and an antagonist of NPY receptors. We have chosen a dose of NPY and of its antagonist against Y1 receptors that were found to be without effects on mean arterial pressure and renal hemodynamics in normoxic rats (Pelayo, et al., 1989; Bischoff, et al., 1997; Bischoff and Michel, 1998). A parenteral route of application of the NPY receptor agonist 12 hours prior to the CO exposure was used to assure constant NPY plasma levels during the 4 hour CO exposure. Interestingly, NPY reversed the enhanced EPO response to 600 ppm CO in DNX rats. Whether NPY can reverse the assumed changes in renal hemodynamics and tubular transport rates (see above) or NPY has a direct inhibitory effect on EPO-secreting cells remains to be established. Correspondingly administration of an NPY receptor antagonist in INN rats increased the EPO-secretory response, although, this increase did not reach significance (Fig. 4).

In contrast, neither beta adrenergic stimulation in DNX nor blockade in INN rats had an effect on EPO secretion. Since Fisher et al. reported decreased EPO concentrations following administration of 4 mg/kg propranolol we have choosen this dose for our experiments in INN rats (Fink, et al., 1975). We administered a subpressoric dose of isoproteronol which has shown not to alter hemodynamics (Pelayo, et al., 1989). In view of the relatively short half-life of isoproteronol in the plasma we administered isoproteronol i.v. via alzet pumps. Thus, the lack of an effect of propranolol and isoproteronol on EPO production in our study is unlikely to be due to an insufficient dose. The data in the literature

concerning the role of beta adrenergic receptors on EPO production are contradictory since it could be shown that a combination of renal denervation and beta-adrenergic receptor blockade inhibited the hypoxia-stimulated EPO secretion (Fink and Fisher, 1976) whereas Jelkmann et al. reported unchanged EPO levels following long-term administration of a selective beta-2 adrenergic agonist (Jelkmann, et al., 1979). Our experiments with beta adrenergic agonists and antagonists argue against a significant role in CO-induced EPO secretion. The differences between our results and those of the literature may reflect differences in the experimental protocols and species.

In the second series of experiments we have chosen to feed rats with a high (HS) and low salt (LS) diet over three weeks to change plasma renin activity (PRA) and thus endogenous angiotensin levels. HS diet exhibited a significant reduced EPO secretory response at 600 ppm CO. This inhibition was completely abolished by bilateral denervation when compared to INN rats on normal diet suggesting that renal innervation is required for the expression of the EPO secretory response to HS diet. Rats kept on LS diet with DNX responded to CO exposure with significantly increased EPO levels as HS rats. In contrast, rats kept on LS diet with intact renal innervation (INN) did not respond with a change of EPO serum levels compared to control rats on normal diet (NS, Fig.5) inspite of very high PRA.

In animal and human studies, elevation of plasma renin activity or administration of angiotensin II was shown to increase EPO secretion (Fisher, et al., 1967; Gould, et al., 1973; Freudenthaler, et al., 1999b; Gossmann, et al., 2001). Furthermore Kato et al. (Kato, et al., 2005) reported an overproduction of EPO in mice harboring the human renin and human angiotensinogen genes. When both genes were introduced into the AT1a receptor null background erythrocytosis was normalized, strongly supporting that angiotensin II was responsible for EPO overproduction. Since these studies are performed under normoxic conditions and since endogeneous angiotensin following LS diet did not increase EPO secretory response in our study we conclude that high PRA and therefore high levels of

endogeneous angiotensin II do not contribute significantly to EPO secretory response following CO exposure in rats.

The rat group (HS) which received a nearly maximal NaCl loading had a very low PRA and most likely very low plasma angiotensin II levels. These rats responded to 600 ppm CO exposure with a reduced EPO secretion. This observation would be in line with the above mentioned findings that angiotensin II can stimulate EPO secretion. The lack of a tight correlation between EPO levels and mRNA in HS rats may be due to (i) simple data scatter, may reflect (ii) a reduced translational efficiency of EPO mRNA or (iii) a diminished half-life of EPO in the blood under this high salt loading condition.

In summary, our data show that renal nerves modulate EPO secretion only at half-maximal CO concentrations. The increase in EPO serum concentrations following exposure to 600 ppm by bilateral renal denervation was reversed by NPY. Therefore our data suggest that renal nerves exhibit an inhibition on EPO producing cells after CO exposure mainly through release of NPY. This notion is supported by the observation that HS-induced reduction of EPO secretion was also blocked by denervation. Rats on LS showed a similar EPO response to denervation as rats on normal salt diet suggesting that high endogeneous angiotensin levels do not contribute to the control of EPO secretion in a LS condition. The mechanism of the inhibition of EPO secretion by release of the renal neurotransmitter NPY remains to be determined.

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Footnotes

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Legends for Figures

Fig. 1: In vivo kinetics of carboxyhemoglobin (HbCO) expressed as percentage of total Hb in response to increasing CO concentrations in inspired air for four hours in conscious rats (n = 5-8 for each CO concentration).

Fig. 2: *In situ* hybridization (ISH) of EPOmRNA in kidneys after 4 hours CO exposure to 1000 ppm (A) and 400 ppm (B) in control (INN) rats (n = 6 per group). Interstitial cells containing EPOmRNA are located in the peritubular interstitial space mainly in the deep cortical juxtamedullary region of the kidney.

Fig. 3: (A) Serum erythropoietin (EPO) and **(B)** Renal EPO mRNA in renal innervated (INN) and denervated (DNX) rats following exposure to increasing CO concentrations (0-1000 ppm) in inspired air (n = 5-8 per group). * $p<0.05$ vs INN following exposure to 600 ppm.

Fig. 4: (A) EPO serum concentrations and **(B)** renal EPO mRNA in rats following renal denervation (DNX) and administration of vehicle (veh), isoproteronol (ISO) or neuropeptide Y (NPY) as well as in innervated rats (INN) with administration of vehicle (veh), propranolol (PROP) or neuropeptide Y1 receptor antagonist (ANPY) following exposure to 600 ppm CO. * $p<0.05$ vs INN+veh, ** $p<0.05$ vs DNX+veh. The number at the bottom of each bar indicates the number of rats in the group.

Fig. 5: (A) EPO serum concentrations and **(B)** renal EPO mRNA in rats on high salt (HS), low salt (LS) diet or normal salt diet (NS) with intact renal nerves (INN) or renal denervation (DNX) following exposure to 600 ppm CO. * $p<0.05$ vs NS in the INN group, ** $p<0.05$ vs

LS in the INN group, *** $p<0.05$ vs HS in the INN group. The number at the bottom of each bar indicates the number of rats in the group.

Figure 1

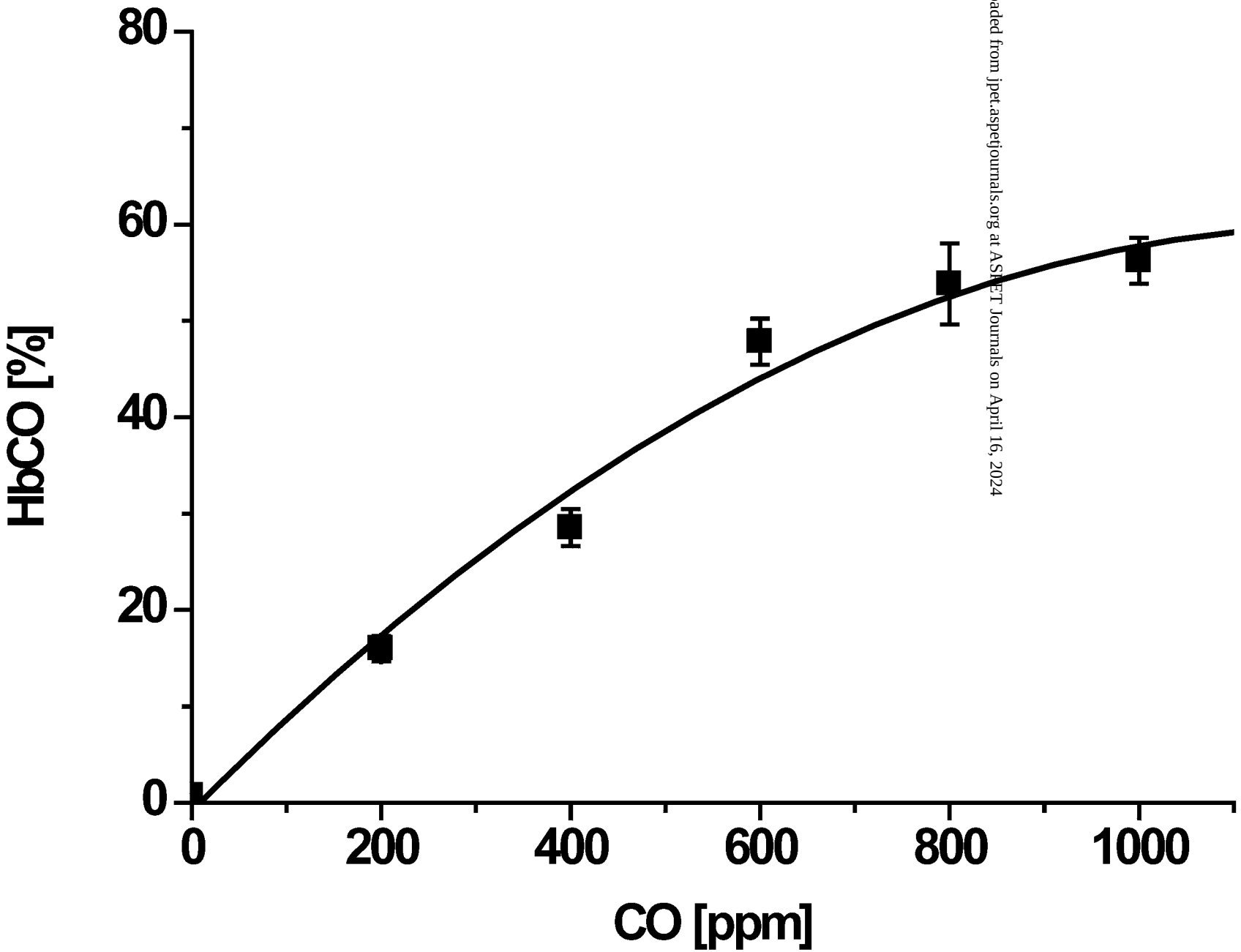
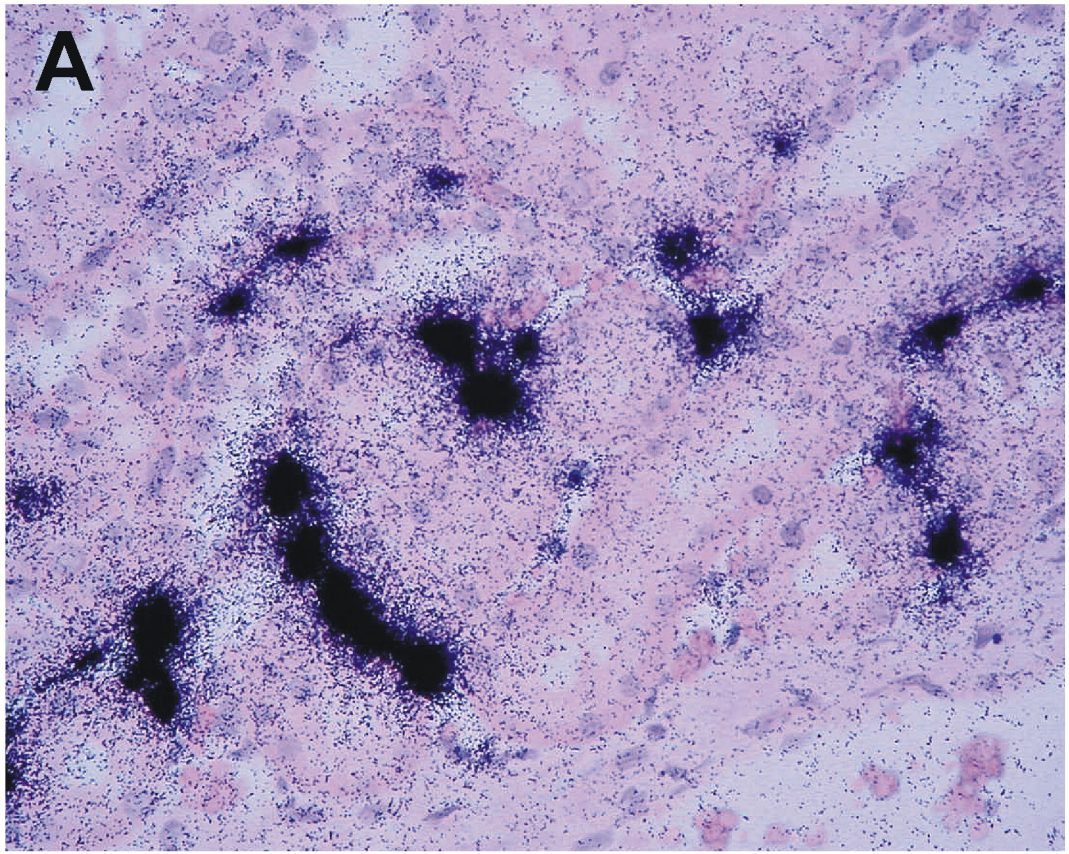


Figure 2



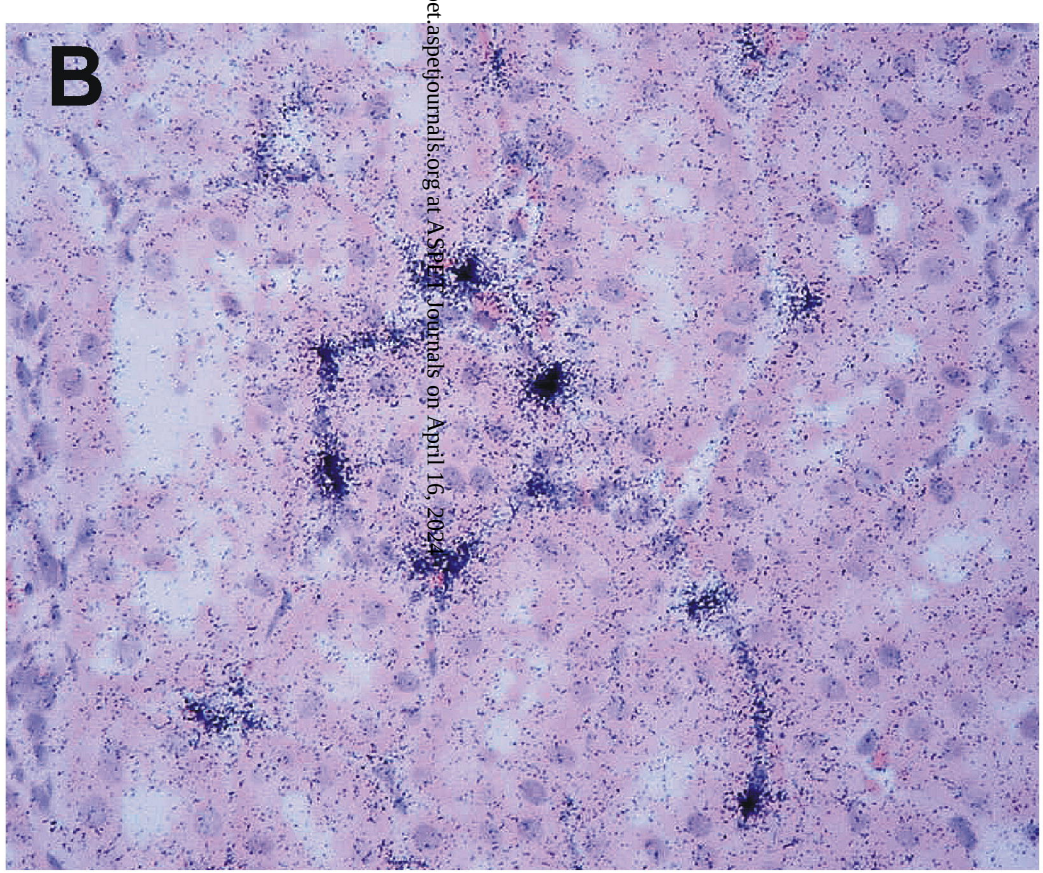


Figure 3

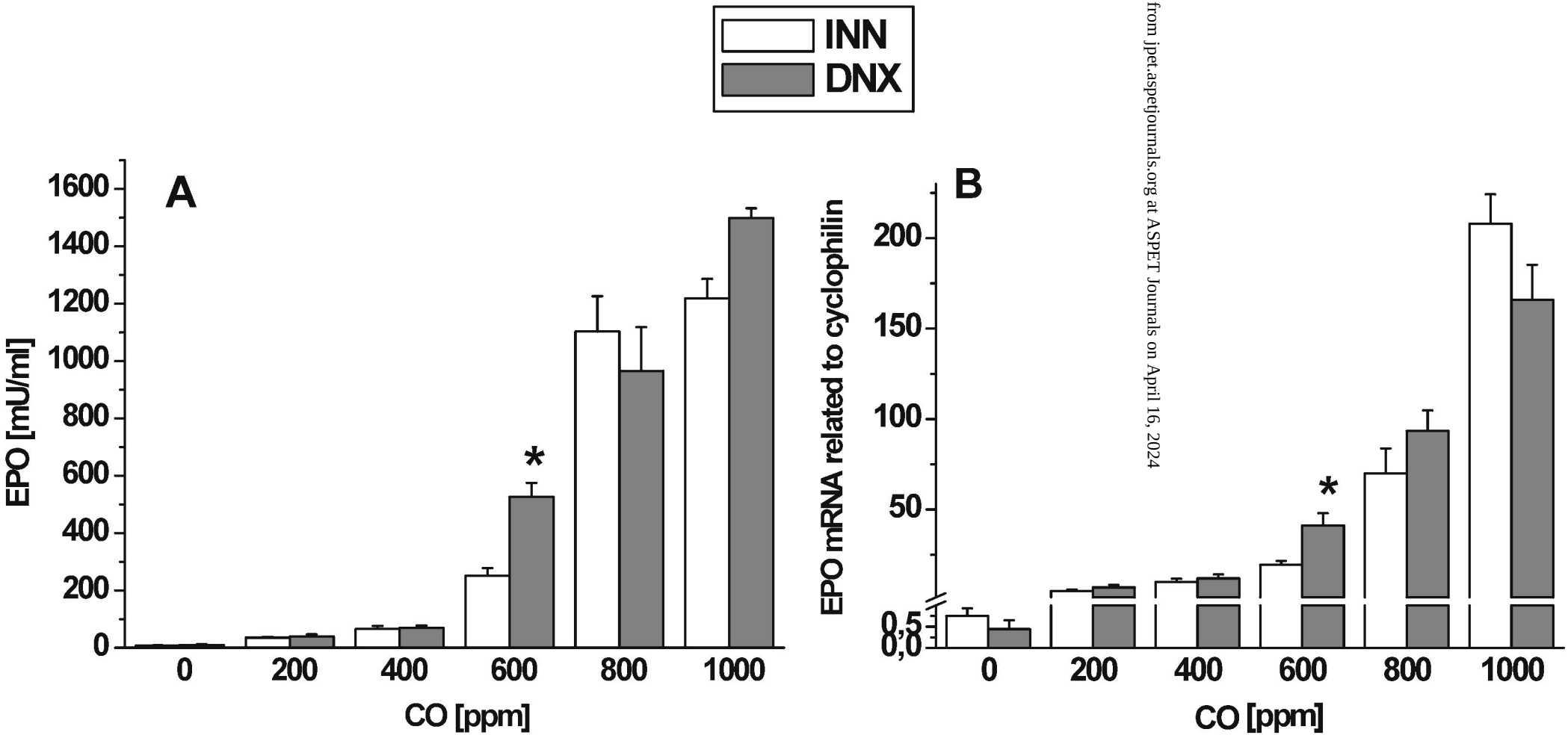


Figure 4

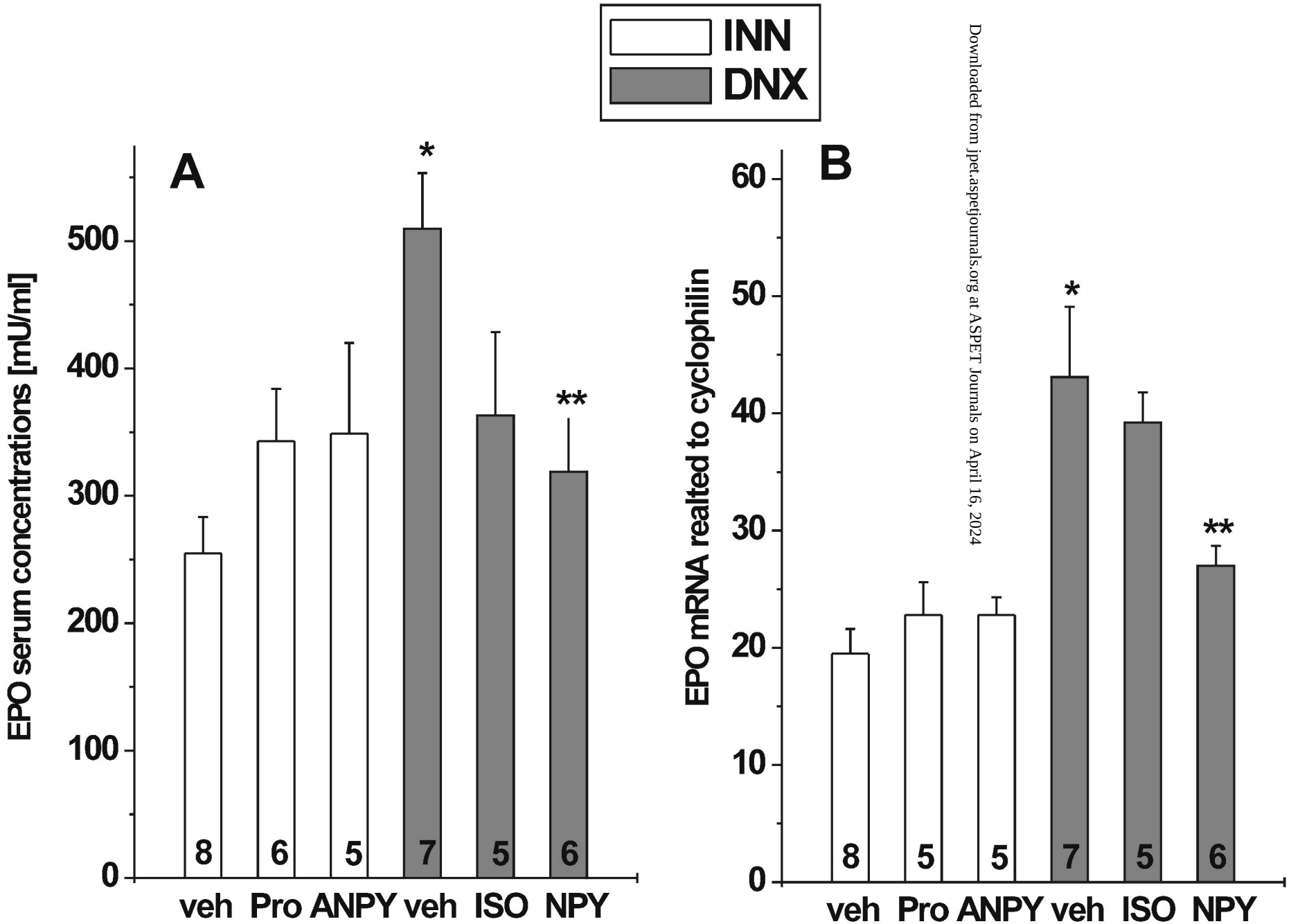


Figure 5