Role of Renal Nerves and Salt Intake on Erythropoietin Secretion in Rats following Carbon Monoxide Exposure

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ABSTRACT

Because the data from the literature contain conflicting results regarding 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 with 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 did not enhance EPO secretion in INN rats after CO exposure. Agonists and antagonists of β-adrenergic receptors had no effect on EPO secretion. High-salt (HS) diet reduced EPO secretory response at 600 ppm CO by 55% compared with INN rats on normal salt diet (p < 0.01). In addition, DNX increased EPO secretion in rats on low-salt 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.
Despite these efforts, the role of renal nerves on EPO secretion remains only poorly understood.

Neuropeptide Y (NPY), another cotransmitter 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 EPO concentrations in a dose-dependent manner. These effects were greatly reduced by administration of the AII receptor antagonist losartan (Freudenthaler et al., 1999a, 2000; Gossmann et al., 2001). However, alteration of endogenous angiotensin II levels following high- (HS) and low-salt (LS) 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).

Because the current data about the role of renal nerves on EPO secretion are equivocal and because the impact of renal nerves on EPO secretion has never been investigated systematically, we reassessed 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 β-adrenergic and NPY receptors on EPO secretion. To alter endogenous AII levels, we chose high- and low-salt diet for 3 weeks as a long-term modulator of inhibition and stimulation of PRA, respectively, of EPO.

### Materials and Methods

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 250 to 320 g were kept on a regular 12-h 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 5 mg/kg i.p. furosemid and subsequent feeding with 0.015% sodium NaCl in drinking water) or on low-salt diet (administration of 5 mg/kg i.p. furosemid and subsequent feeding with 0.015% sodium chow) for 3 weeks. The rat experiments were conducted in accordance with the German Animal Protection Law.

**Experimental Design and Groups.** Seven days following bilateral renal DNX, rats were exposed to different CO concentrations. To change the endogenous angiotensin II concentration in the plasma, we studied the effect of DNX and low-salt (LS + DNX) diet compared with controls without DNX (HS and LS) before exposure to 600 ppm CO. Rats were sacrificed after 4 h of CO exposure for taking blood samples and harvesting kidneys, which were stored at −80°C until analysis.

**Renal Bilateral Denervation.** Bilateral renal DNX was carried out in anesthetized rats with 70 mg/kg b.w. ketamine i.p. (Parke-Davis, Freiburg, Germany) and 15 mg/kg b.w. xylazine i.p. (Bayer, Leverkusen, Germany) as described previously (Mühlbauer 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 right renal hilus for 2 to 4 min without destruction of renal nerves. Completeness of DNX was assessed by measuring renal norepinephrine content with high-pressure liquid chromatography (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 anesthetized 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; Direct Corporation, Cupertino, CA) preloaded with the appropriate solution, was inserted. A polyethylene catheter fitted to the drug delivery portal of the osmotic minipump was inserted into the external jugular vein. Wounds were closed with nonreabsorbative material (Vicryl 3/0; Ethicon, Brüssel, Belgium).

**CO Experiments.** CO was mixed with room air (inflow between 6 and 10 l/min) to generate CO concentrations between 200 and 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 h of CO exposure, and blood was collected into serum tubes for determination of hemocrit, 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.

**Chemical Analysis.** Serum EPO concentrations were determined by enzyme-linked immunosorment assay with a commercially available kit (Medac, Wedel, Germany). Arterial blood samples were analyzed for hemocrit. PRA was determined by its capacity to generate 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.

**Real-Time Polymerase Chain Reaction.** 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). PCR was carried out on the LightCycler instrument with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Primer sequences used for amplification were as follows: EPO (RefSeq accession no. NM_017001): sense, 5'-GACATTGGCTTCCAGGTATT-3' and antisense, 5'-GGCCTGGTTCAGGTATT-3; and peptidyl prolly isomerase A (PPIA) (NM_017101): sense, 5'-GGGAGCAAAGGATTGCTGA-3' and antisense, 5'-GGGAGCAAAGGATTGCTGA-3. PPIA (cyclophilin A) served as internal control. Standard samples were included for comparison among different PCR runs. The relative expression ratio of the target gene EPO was calculated according to Pfaffl (2001).
In Situ Hybridization. Kidneys of control rats (INN) obtained after 4 h CO exposure to 400 and 1000 ppm were fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer, pH 7.2, overnight and embedded in paraffin. Five-micrometer tissue sections were dewaxed and hybridized as described previously (Klingel et al., 1992). Hybridization probes at a length of 502 base pairs were generated by in vitro transcription of a mouse EPO cDNA cloned into pGEM-3Z SP6 using T7 polymerase (antisense). The hybridization mixture [10 mM Tris-HCl, pH 7.4, 50% (v/v) deionized formamide, 600 mM NaCl, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll-Paque Plus, 0.05% bovine serum albumin, 10% dextran sulfate, 10 mM dithiothreitol, 200 μg/ml denatured sonicated salmon sperm DNA, and 100 μg/ml rabbit liver tRNA] contained either the 35S-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 42°C for 18 h. After washing steps, the slide preparations were exposed at 4°C for 3 weeks. After development, the slides were stained with hematoxylin and eosin.

Chemicals. All chemicals, the neuropeptide Y agonist [Leu31,Pro34]-NPY, the neuropeptide Y1 receptor antagonist Ile-Glu-Pro-Dapa-Tyr-Arg-Leu-Arg-Tyr-NH2, cyclic (2,4’2’,4’) diamide (ANPY), PROP, and 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. All values are presented as mean ± S.E.M. p < 0.05 was considered to be statistically significant.

Results

Degree of Hypoxia. To characterize our model of systemic deficiency of oxygen delivery, we measured CO binding to hemoglobin in rats exposed to increasing CO concentrations in air (n = 5–8/group). As shown in Fig. 1, increased concentrations of CO in inspired air for 4 h led to an increase of HbCO up to 56% after exposure to 1000 ppm CO. The increased HbCO concentration enhances sharply the O2 binding affinity of hemoglobin and thus reduces the O2 delivery to the tissue (Coburn and Forman, 1987). Our in vivo data are in good agreement with those of Coburn and Forman (1987). Hematocrit values were between 44 and 48% after CO exposure in all groups.

To localize the cell types in kidneys that express EPO mRNA after 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 indicated EPO mRNA of the interstitial kidney cells was much higher following severe hypoxia (1000 ppm; Fig. 2A) compared with that of 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 35S-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 with baseline concentrations without a difference between rats with or without renal denervation (Fig. 3B). EPO serum levels in DNX rats, however, were 2-fold higher following exposure to 600 ppm (527 ± 48 AU/ml) compared with INN rats (250 ± 41 AU/ml; p < 0.05; Fig. 3A), indicating a higher sensitivity of DNX rats to CO, which stimulates EPO secretion half-maximally. 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 with 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 [Leu31,Pro34]-NPY resulting in similar EPO serum concentrations compared with 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 NPY1 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. 4, A and B). Application of isoproterenol in DNX rats as well as propranolol in INN rats showed an insignificant tendency to decrease or increase EPO serum concentrations, respectively (Fig. 4A). Isoproterenol 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. HS diet reduced EPO secretory response by 55% at 600 ppm CO compared with rats on normal salt (NS) diet (p < 0.01; Fig. 5A). However, this inhibitory action of HS was greatly attenuated by DNX with 3-fold higher EPO serum levels compared with 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). LS diet did not significantly change 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 ng of AI/ml/h) was significantly decreased compared with rats on NS (19.2 ± 1.6). PRA showed a 5.6-fold increase (107.7 ± 6.8 ng of AI/ml/h) in rats after 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 ng of AI/ml/h, respectively).

Fig. 1. In vivo kinetics of HbCO expressed as percentage of total Hb in response to increasing CO concentrations in inspired air for 4 h in conscious rats (n = 5–8 for each CO concentration).
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 with controls. Exposure of rats to hypoxia was performed 7 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 (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 (Mazzola et al., 2005; Moore 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 signaling, 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, after exposure to different CO concentrations, was similar in INN and DNX rats. However, DNX rats revealed a significant higher EPO secretory response after exposure to 600 ppm compared with INN rats. In previous studies, animals were exposed to only severe and nearly maximal hypoxic stimuli (1000 ppm, 10% \( \text{O}_2 \) or 0.42 atmospheres over 4, 6, or 5 h, respectively) (Fink and Fisher, 1976; Beynon, 1977; Eckardt et al., 1992). 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 after exposure to 1000 ppm CO in our study is in good agreement with the increases reported by Eckardt et al. (1992). The lack of an effect of renal denervation after exposure to 1000 ppm further suggests that this severe hypoxic stimulus with \( \text{HbCO} \) 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. (1975). The difference of EPO secretory response after 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 glomerular filtration rate to the same extent as innervated kidneys. Besides catecholamines, NPY also is a potent renal vasoconstrictor (Bischoff and Michel, 1998). Because a higher glomerular filtration rate imposes to the tubular system a higher workload for electrolyte reabsorption, renal interstitial oxygen partial pressure may be even lower in DNX compared with 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 after ischemia (Recordati et al., 1981; Bischoff and Michel, 1998). Because 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 7 days may have discrete effects on central nervous system-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 was found to be without effect 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 h before the CO exposure was used to ensure constant NPY plasma levels during the 4-h 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. (1975) reported decreased EPO concentrations following administration of 4 mg/kg propranolol, we have chosen this dose for our experiments in INN rats. We administered a subpressor dose of isoproterenol, which has been shown to not alter hemodynamics (Pelayo et al., 1989). Because of the relatively short half-life of isoproterenol in the plasma, we administered isoproterenol i.v. via Alzet pumps. Thus, the lack of an effect of propranolol and isoproterenol on EPO production in our study is not likely to be due to an insufficient dose. The data in the literature concerning the role of \( \beta \)-adrenergic receptors on EPO production are contradictory, because 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. (1979) reported unchanged EPO levels following long-term administration of a selective \( \beta_2 \)-adrenergic agonist. 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 chose to feed rats with an HS and LS diet over 3 weeks to change PRA and thus endogenous angiotensin levels. HS diet exhibited a significant reduced EPO secretory response at 600 ppm CO. This inhibition was abolished by bilateral denervation compared with 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 with control rats on NS diet (Fig. 5), in spite 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; Gosmann et al., 2001). Furthermore, 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 erythropoiesis was normalized, strongly supporting that angiotensin II was responsible for EPO overproduction. Since these studies are performed under normoxic conditions and since endogenous angiotensin following LS diet did not increase EPO secretory response in our study, we conclude that high PRA and therefore high levels of endogenous angiotensin II do not contribute significantly to EPO secretory response following CO exposure in rats.

The rat group that received a nearly maximal NaCl loading (HS) 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 1) simple data scatter or 2) reflect a reduced translational efficiency of EPO mRNA or 3) 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 endogenous 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.

References

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