Brief Definitive Report

Oxygen-Dependent Modulation of Erythropoietin mRNA Levels in Isolated Rat Kidneys Studied by RNase Protection

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Summary

Using oligonucleotide primers complementary to conserved regions in the mouse erythropoietin (Epo) gene, a portion of the rat Epo gene was amplified by the polymerase chain reaction to produce a probe suitable for assay of rat Epo mRNA by RNAse protection. The assay, which has sufficient sensitivity to measure to Epo mRNA in unstimulated rat kidneys, was used to demonstrate high amplitude in vitro modulation of Epo mRNA levels in response to changes in perfusate flow rate and oxygen tension in isolated kidneys, thus providing clear evidence that all the necessary events linking changes in oxygen delivery to the modulation of Epo mRNA levels can occur intrarenally.

In early experiments, accumulation of erythropoietin (Epo) in the perfusate of isolated kidneys provided important evidence that the kidney is the site of synthesis (1, 2). These experiments also suggested that the sensing mechanism was intrarenal, since, in some studies, hypoxic perfusion was associated with an increased accumulation of Epo in the perfusate. Recent work (3–5) has shown that a major control mechanism for Epo synthesis operates by modulation of Epo mRNA, mediated at least in part at the transcriptional level (5). We therefore argued that if all components forming the major link between blood oxygen availability and Epo synthesis were intrarenal, then it should be possible to show modulation of mRNA levels in isolated kidneys and to use such a system to study the physiology of the sensing mechanism. Unfortunately, studies using the relatively insensitive method of Northern blotting have not been able to demonstrate Epo mRNA in unstimulated kidneys, requiring investigators to apply relatively severe stimuli in vivo to bring Epo mRNA levels into a detectable range (3–5).

To address this problem, we have used a sensitive RNAse protection assay (6) for rat Epo mRNA. In this paper, we describe the detection of Epo mRNA in unstimulated rat kidneys, the quantitation of mRNA levels using β cytoplasmic actin as an internal control, and the application of this method to the isolated perfused rat kidney to demonstrate in vitro modulation of mRNA levels in response to changes in oxygen delivery.

Materials and Methods

Kidney Perfusion. Isolated kidneys from male Wistar rats (330–390 g) were perfused in a recirculating circuit using modifications of the technique described by Ross et al. (7). Kidneys were perfused for 2 h at constant perfusate flow, which was achieved through feedback control of the perfusion pressure. The perfusate consisted of Krebs-Henseleit buffer containing 3 or 5% BSA (Fraction V; Miles Laboratories, Slough, UK) supplemented with washed human erythrocytes (final haematocrit 0.11–0.16, glucose, 5 mmol/liter), and the 20 physiological amino acids. ^{14}C-inulin (Amersham International, Amersham, UK) was added to permit measurement of glomerular filtration rate by inulin clearance. Oxygen tensions were measured using an ABL-3 blood gas analyzer (Radiometer, Copenhagen, Denmark). All experiments were performed with erythrocytes from a single donor. Hemoglobin-bound oxygen content (μmol/ml) was calculated using the formula: $0.62 \times$ [saturation (fractional) $\times$ hemoglobin (g/dl)]. The dissolved oxygen content of the perfusate was calculated according to the formula: O_2 content (μmol/ml) $= 1.257 \times 10^{-3} \times$ [pO$_2$ (mmHg) $\times$ (1-haemacrit].

RNAse Protection Assay. Kidneys were homogenized in guanidine thiocyanate, and RNA was purified on a cesium chloride gradient. The fully complementary genomic probe used as the template for production of the labeled RNA transcripts necessary in the RNAse protection assay was obtained by amplification of rat genomic DNA using oligonucleotides complementary to conserved regions in exons IV and V of the mouse sequence in the PCR. From the amplification product, a PstI/SacI fragment, containing 132 bp of exon V and ~300 bp of the intron extending 5′ to this sequence, was constructed and inserted into pSP64 for generation of the RNA transcripts. A partial nucleic acid sequence of this probe was determined by the dideoxy chain termination method. The actin probe was constructed from a genomic clone of the rat cytoplasmic β-actin gene, which was generously donated by Dr. Uri Nudel of the Weizman Institute of Science, Rehovot, Israel. An AvaI/HindIII fragment containing the 76 nucleotide first exon and ~200 bp of surrounding sequence was inserted into pAM19. Sample

RNA was analyzed by an RNAse protection assay (6). To ensure equivalent specific activity, actin and erythropoietin probes used on a single sample of RNA were always made from the same source of α[^{32}P]GTP. For analysis of erythropoietin mRNA, an aliquot of total RNA was dissolved in hybridization buffer consisting of 80% formamide, 40 mmol/l Pipes, pH 6.4, 400 mmol/liter NaCl, and 1 mmol/l EDTA. Hybridization was performed at 60°C using a 50-μl aliquot, containing 250 μg total RNA and 0.5-1 × 10^6 cpm radiolabeled erythropoietin probe. RNAse digestion was performed at 20°C for 30 min, and the protected fragments were run on a denaturing 10% polyacrylamide gel. For normalization to the β-cytoplasmic actin signal, an aliquot of original solution of RNA in hybridization buffer was further diluted to a concentration of 0.05 μg/μl, and a parallel hybridization was performed at 60°C using a 50-μl aliquot, containing 2.5 μg total RNA and 2-2.5 × 10^6 cpm radiolabeled actin probe. After RNAse digestion at 20°C for 30 min, one-tenth of the products were added to the erythropoietin analysis so that subsequent purification and gel electrophoresis were performed together. X-ray film was exposed to the dried gels at −70°C, after which the protected bands were excised from the gel, set in solid scintillant, and counted using a flat matrix β counter (1205; Pharmacia/LKB, Inc., Gaithersburg, MD). Kendall's tau was calculated to test the significance of associations between variables.

Results

DNA sequence analysis of the rat genomic erythropoietin probe demonstrated a very high degree of homology with the mouse, and the mobility of the protected fragment was exactly that expected from conservation of the splice site between exons 4 and 5.

Using 250 μg of total RNA, erythropoietin mRNA was readily detected in kidneys from normal animals. Comparison with the signal from β cytoplasmic actin mRNA showed, nevertheless, that the level of erythropoietin mRNA was very low in these unstimulated kidneys. Allowing for the different guanosine contents of the protected erythropoietin probe fragment and actin fragment, the ratio of erythropoietin mRNA to β cytoplasmic actin mRNA is ~1:10,000. During 2 h of isolated kidney perfusion, the level of actin mRNA did not change, and this was used as an internal control for the semi-quantitative estimate of Epo mRNA.

Demonstration of Modulation of Epo mRNA in Isolated Kidneys. During isolated perfusion for 2 h with medium containing erythrocytes at a hematocrit of 11-16%, a striking increase in Epo mRNA was seen under appropriate conditions. To be sure that the stimulation of Epo mRNA observed in perfused kidneys did not occur during surgical dissection for the perfusion nephrectomy, the contralateral left kidney was removed and homogenized for RNA extraction immediately after commencing perfusion of the right kidney. These kidneys contained similar levels of Epo mRNA to those in kidneys taken immediately from unstimulated animals.

To analyze some characteristics of the in vitro response of

Table 1. *Functional Parameters for Isolated Kidneys*

	Epo mRNA										
	Base-line	Perfused kidney	Flow	Hct (fractional)	PAO$_2$	O$_2$ delivery	pVO$_2$	Venous saturation	O$_2$ consumption	GFR	F$_R$Na
Exp.											
	× 10^{-4}		ml/min		mmHg	μmol/min	mmHg	%	μmol/min	ml/min	%
1	–	21.4	4	0.16	152	15.5	56	87	2.03	0.55	86.4
2	–	15.4	5	0.16	164	17.8	61	89	2.24	–	–
3	0.54	9.12	5	0.13	171	14.4	57	87	2.09	0.17	80.0
4	0.33	26.2	5	0.13	158	14.2	72	93	1.28	–	–
5	1.16	9.84	12	0.15	158	37.0	68	93	3.29	0.70	87.6
6	1.37	6.40	15	0.12	171	40.7	68	93	4.00	0.73	88.3
7	0.82	2.79	18	0.11	148	43.7	62	89	5.85	1.51	90.3
8	1.31	2.46	20	0.16	140	71.3	70	93	5.64	1.76	75.7
9	0.69	0.58	25	0.13	143	74.0	73	93	6.19	0.79	98.6
10	1.57	9.04	5	0.15	374	28.4	81	94	2.55	–	–
11	0.48	1.58	12	0.13	367	36.8	160	99	3.03	0.73	99.0
12	0.67	0.19	12	0.15	427	42.7	174	99	3.64	–	–
13	0.64	1.04	15	0.16	423	54.9	152	99	4.88	1.18	93.2
14	0.59	0.31	18	0.15	406	63.5	160	99	5.46	1.52	86.1
15	1.03	0.33	20	0.11	387	53.6	163	99	5.31	1.07	92.4

The Epo mRNA values are the mean of duplicate measurements and refer to the abundance relative to Actin mRNA. Epo mRNA was negatively associated with perfusate flow rate in both groups ($p < 0.05$). Oxygen consumption was positively associated with perfusate flow rate ($p < 0.05$) in both groups.

Epo mRNA to oxygen delivery, 15 experiments were performed at different perfusate flow rates and oxygen tensions (Table 1). In all kidneys, renal oxygen delivery and consumption were stable for duration of the experiment. The Epo mRNA to β Actin mRNA ratio was $0.86 \pm 0.39 \times 10^{-4}$ SD in the contralateral control kidneys. The modulation of Epo mRNA was dependent on perfusate flow rate (Table 1). Fig. 1 shows the results for four experiments conducted in the low pO$_2$ range, using 20% O$_2$, 75% N$_2$, 5% CO$_2$ to gas the perfusate. The 20–40-fold increase in Epo mRNA that occurred at a perfusate flow rate of 5 ml/min was reduced as perfusate flow rate was increased. In the high pO$_2$ range obtained by gassing the perfusate with 95% O$_2$, 5% CO$_2$, only the lowest flow rate studied caused significant stimulation of Epo mRNA. Fig. 2 shows the RNA analysis from two pairs of kidneys perfused at the similar flow rates but high or low oxygen tensions, and demonstrates the dependence of Epo mRNA modulation on perfusate pO$_2$. In kidneys perfused at high flow rate and high oxygen tension, Epo mRNA was lower than in the contralateral control kidney.

Discussion

This assay was capable of detecting Epo mRNA for the first time in unstimulated rat kidneys, a result in keeping with the existence of a basal circulating level of the hormone and the established view of the kidney as the principal site of synthesis.

The demonstration of high amplitude modulation of erythropoietin mRNA levels in isolated kidneys provides clear evidence that all the necessary events linking changes in oxygen delivery to the modulation of Epo mRNA levels can occur intrarenally. This result is consistent with the demonstration of oxygen-dependent synthesis of erythropoietin by certain cell lines derived from malignant tissues, notably hepatoma (8), and is also consistent with the results of studies on isolated perfused kidneys in which a greater accumulation of erythropoietin was observed during hypoxia (2, 9). One difficulty, however, with measurement of erythropoietin production itself in isolated kidney perfusion experiments, has been that the time required to accumulate measurable quantities in the perfusate is long in relation to the viability of the preparations. Modulation of Epo mRNA, since it occurs earlier, allows reduction of the perfusion time to a period well within the limits of stability of this preparation. Furthermore, by measurement of Epo mRNA in the contralateral kidney at the start of perfusion, it was possible to prove that
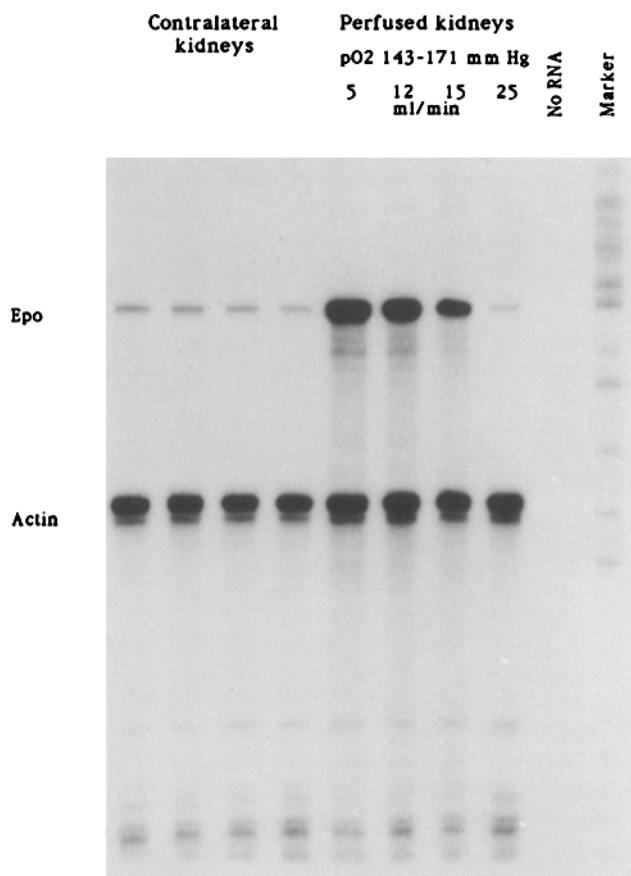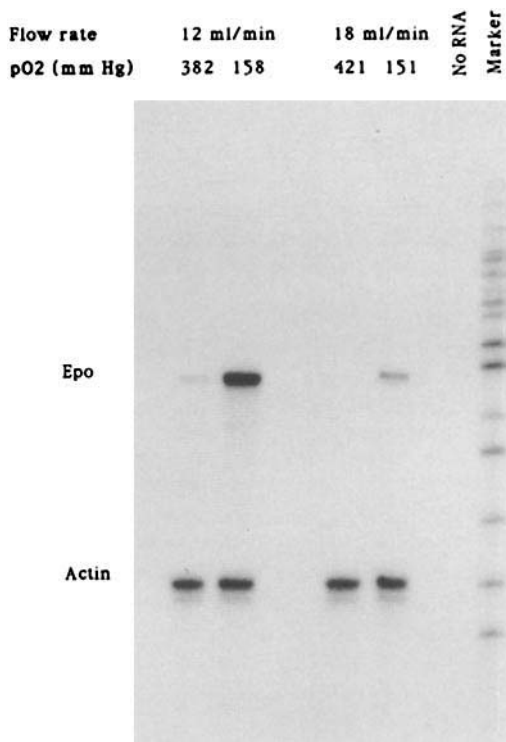

Figure 1. RNAse protection analysis of mRNA from four isolated kidneys perfused at oxygen tensions in the lower range (143–171 mmHg), but at different flow rates. The four lanes on the left contain mRNA from the contralateral (unstimulated) kidney removed at the start of perfusion. The Epo signal arises from 250 μg total RNA, and the actin signal arises from 250 ng total RNA. Size markers were generated by a HpaII digest of pBR322 DNA. The autoradiograph was exposed at $-70°C$ for 14 h.

Figure 2. RNAse protection analysis of mRNA from isolated kidneys. Pairs of kidneys were perfused at similar flow rates but different oxygen tensions. The Epo signal arises from 250 μg total RNA, and the actin signal arises from 250 ng total RNA. The autoradiograph was exposed at $-70°C$ for 8 h.

modulation of Epo mRNA occurred during isolated perfusion and not during the dissection for perfusion nephrectomy.

The responses of the perfused kidney to changes in perfusate pO_2 and perfusate flow rate are consistent with in vivo regulation of erythropoietin synthesis, although reduction in erythropoietin synthesis by increasing renal blood flow has not been described in vivo. In a recent study on the rat, it was shown that reduction of renal blood flow by constriction of the renal artery increased erythropoietin synthesis, to a much lesser extent than anemia (10). The authors of that study offered two explanations for the result, first, that the major mechanism linking Epo production to blood oxygen availability might require an extrarenal component, and second, that the changes in intrarenal oxygenation might differ between the two stimuli because of the interdependence of changes in renal blood flow and renal oxygen consumption. Our results strongly favor the latter explanation. Although a reduction in Epo mRNA was observed at increased perfusate flow rate despite increased renal oxygen consumption, in terms of total oxygen delivery, increasing perfusate flow rate was less effective in suppressing Epo mRNA than increasing the perfusate oxygen tension.

It is of interest that modulation of Epo mRNA took place when venous oxygen saturation was in the range of 85-94%. The existence of areas of profound intrarenal hypoxia most probably dependent on inhomogenous oxygen delivery arising from shunt diffusion of oxygen has been appreciated for some time (11), and may provide an explanation for this finding if, as is widely believed and supported by experiments on hepatoma cells (8), tissue hypoxia is the immediate stimulus.

References

1. Fisher, J.W. and B.J. Birdwell. 1961. The production of an erythropoetic factor by the *in situ* perfused kidney. *Acta Haematol. (Basel).* 26:224.
2. Zangheri, E.O., H. Campana, F. Ponce, J.C. Silva, F.O. Fernandez, and J.R.E. Suarez. 1963. Production of erythropoietin by anoxic perfusion of the isolated kidney of a dog. *Nature (Lond.).* 199:572.
3. Schuster, S.J., J.H. Wilson, A.J. Erslev, and J. Caro. 1987. Physiologic regulation and tissue localization of renal erythropoietin messenger RNA. *Blood.* 70:316.
4. Beru, N., J. McDonald, C. Lacombe, and E. Goldwasser. 1986. Expression of the erythropoietin gene. *Mol. Cell. Biol.* 6:2571.
5. Schuster, S.J., E.V. Badiavas, P. Costa-Giomi, R. Weinmann, A.J. Erslev, and J. Caro. 1989. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. *Blood.* 73:13.
6. Melton, D.A., P.A. Kreig, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of

biological active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035.
7. Ross, B.D., F.H. Epstein, and A. Leaf. 1973. Sodium reabsorption in the perfused rat kidney. *Am. J. Physiol.* 225:1190.
8. Goldberg, M.A., S.P. Dunning, and H.F. Bunn. 1988. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science (Wash. DC).* 242:1412.
9. Eckardt, K.-U., A. Kurtz, H. Scholz, and C. Bauer. 1989. Regulation of erythropoietin formation *in vivo. Contrib. Nephrol.* 76:33.
10. Pagel, H., W. Jelkmann, and C. Weiss. 1988. A comparison of the effects of renal artery constriction and anaemia on the production of erythropoietin. *Pfluegers Arch. Eur. J. Physiol.* 413:62.
11. Brezis, M., S. Rosen, P. Silva, and F.H. Epstein. 1984. Renal ischemia: a new perspective. *Kidney Int.* 26:375.