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An ESR contrast agent is transported to rat liver through organic anion transporter

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Abstract

Carboxy PROXYL is a useful extracellular paramagnetic contrast reagent in electron spin resonance (ESR) and magnetic resonance imaging (MRI). Active transfer of the probe was investigated using an *in situ* liver model in rats. Carboxy PROXYL, a nitroxyl spin probe, was perfused into *in situ* liver perfusion system from Wistar rats. Concentration of nitroxyl form of the spin probe in effluent increased gradually after introducing perfusate with the spin probe and reached a plateau. The disappearance of Carboxy PROXYL from the perfusate was 40%, which could not be explained with its partition coefficient. Administration of non-selective inhibitors of organic anion transporters, *p*-aminohippuric acid and penicillin G, inhibited competitively and in a dose dependent manner the transfer of Carboxy PROXYL into rat liver *in situ*, resulting in increases of Carboxy PROXYL in the effluent. The results demonstrate that there is an active transfer system of an ESR contrast reagent into *in situ* rat liver through organic anion transporters.

Keywords: *In situ* liver perfusion, electron spin resonance spectroscopy, spin probe, rat, organic anion transporter, nitroxyl radical

Introduction

Electron spin resonance (ESR) is a magnetic resonance spectroscopic and imaging technique that measures species containing unpaired electrons. The ESR technique has been applied to measure oxidative stress *in vivo* non-invasively using stable nitroxyl radical which are both ESR imaging probes and antioxidants [3,7,16,18,22]. It is important to choose an optimal contrast agent(s) to measure oxidative stress or obtain image contrast in select regions of interest. One important factor in a contrast reagent is reported as its partition co-efficient, which may influence its distribution in tissues.

One of the most important hepatic functions is to clear metabolites or xenobiotics. There are several systems reported which account for the clearance, which include organic anion transporter (OATs). OATs is a family of multi-specific transporters, which transports a variety of structurally unrelated compounds but having negative charge [9,17]. Much attention has been directed to identify and characterize the transporters in last decade [12,15].

Recently, confocal microscopic technique was applied to the imaging of organic anion transports in renal proximal tubule cells [8], or in rat choroids plexus with fluorescent organic anion fluorescein [1]. Fluorescence labeling technique is, however, difficult to be applied to a measurement deep inside body without an invasive operation and would be limited in the application to *in vitro* or *in situ* experiments. In the

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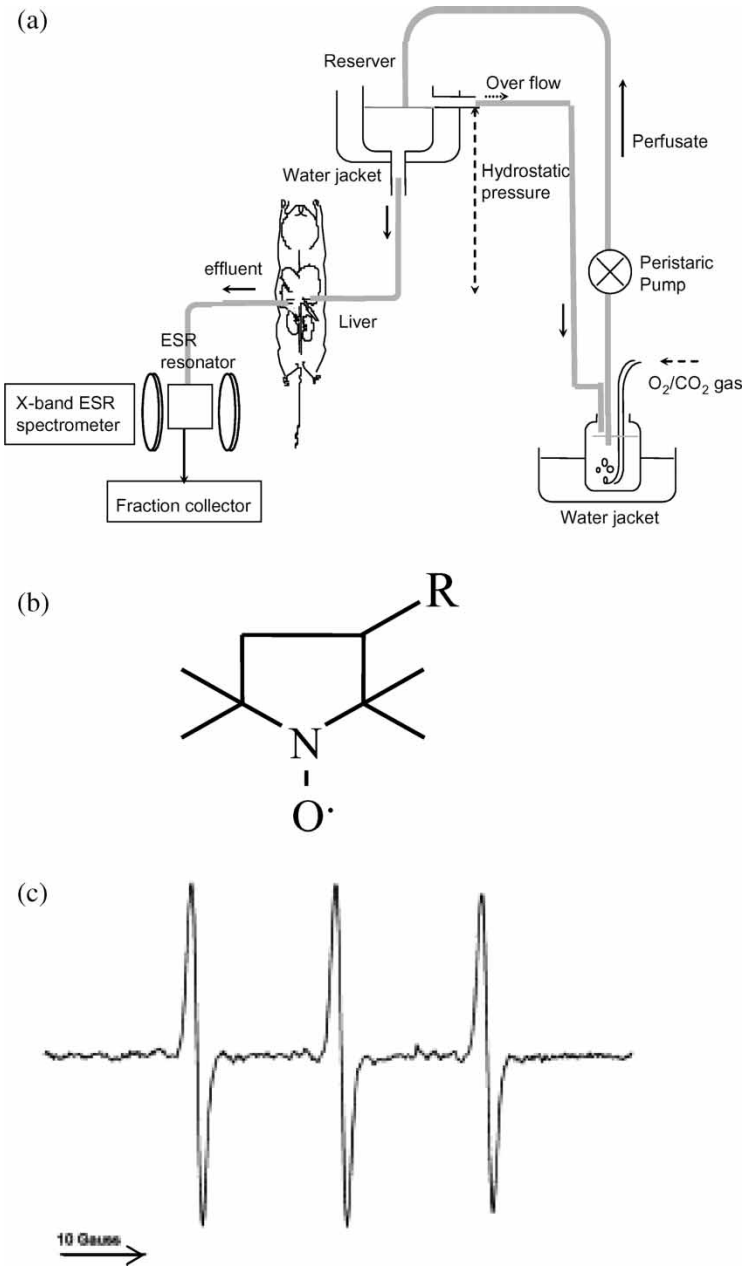


Figure 1. Experimental setup and the spin probes used in this study: (a) schematic diagram of the experimental setup for rat liver perfusion *in situ*; (b) chemical structure of the spin probes, R=COO^- for 3-carboxy-2,2,5,5,-tetramethylpyrrolidine-1-oxyl (Carboxy PROXYL), R=$CONH_2$ for 3-carbamoyl-2,2,5,5,-tetramethylpyrrolidine-1-oxyl (Carbamoyl PROXYL), (c) typical X-band ESR spectra of 10 μM Carboxy PROXYL dissolved in Krebs–Henseleit bicarbonate buffer. ESR spectra were measured as described in "Materials and methods" section.

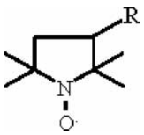
current study, we found a rat liver uptake of an ESR contrast reagent, 3-carboxy-2,2,5,5,-tetramethylpyrrolidine-1-oxyl (Carboxy PROXYL), *in situ* through OATs.

Material and methods

Chemicals

Carboxy PROXYL, 3-carbamoyl-2,2,5,5,-tetramethylpyrrolidine-1-oxyl (Carbamoyl PROXYL), basic structure of which was shown in Figure (1b), was purchased from Aldrich (St Louis, MO). Reported property of these spin probes were summarized in Table I [2,10,16]. All other reagents were of highest grade commercially available. Final concentrations of spin probes in the perfusate were prepared as 0.01 mM. Animal breeding and care were carried out according to our institutional animal research guideline. Wistar male rats (6-weeks old) were purchased from Seac Yoshitomi Co. (Fukuoka, Japan) and were kept in 12 h dark/light cycles with *ad libitum* intake of water and chow (MF, Oriental Yeast Co. Tokyo, Japan).

Table I. Physical properties of nitroxyl spin probes used in this study.

Spin probe		*n*-octanol/water
	R=$CONH_2$	0.58 [0.68]
	R=COO^-	0.0032 [0.01]

N-octanol/water (pH 7.4) partition coefficients. All values represent the mean of at least two experiments. [] Values are obtained from references.

In situ liver perfusion system

In liver organ perfusion experiments, cellular function, hepatocytes polarity or circulatory systems are intact, which enable us to investigate drug transport and metabolism in liver quantitatively under physiological condition [13]. *In situ* rat liver perfusion was carried out for the study of hepatic distribution and metabolism of nitroxyl spin probes under physiological integrity, since the metabolism of nitroxyl spin probes or hepatic functions might be affected by nervous or hormonal control [14]. A preparation of *in situ* liver perfusion system is shown in Figure (1a). Krebs–Henseleit buffer (KHB) were saturated with O_2/CO_2 gas (95%/5%) and was adjusted as pH 7.4. Wister rats were anesthetized with Nembutal (75 mg/kg body weight), then *in situ* liver perfusion were carried out as reported in the literature [13]. A silicone tubing for the perfusate was inserted into portal vein. KHB was incubated at 37°C and perfused at 4 ml/min/g liver into the portal vein. The proper hepatic artery was ligated with a nylon thread. Then, inferior vena cava was clipped to discharge blood, which was then ligated to shunt the flow. A tubing was connected to bile duct to collect the bile for further analysis. Outlet tubing for the perfusate was inserted to the thoracic inferior vena cava, such that perfusate running from the portal vein was guided to the outlet tubing at the thoracic inferior vena cava. The effluent from the outlet tubing was directly introduced into quartz capillary in a X-band ESR spectrometer (JES-RE-1X, JEOL, Japan). The outflow from the capillary was collected with a fraction collector for further analysis.

After pre-perfusion by using KHB without spin probe for 30 min, KHB containing the spin probe being examined was perfused and its ESR spectra were continually observed. The spin probe level was monitored using ESR line at the lower magnetic field of the triplet lines of the spin probe. Standard sample of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to quantify the amount of spin probes from the ESR signal heights. For competitive inhibition experiments with *p*-aminohippuric acid or penicillin G, the inhibitor was added both in pre-perfusion buffer and perfusion buffer with the spin probe. The magnetic field was 336 ± 7.5 mT. The microwave frequency was 9.44 GHz and microwave power was 5.0 or 10.0 mW. The amplitude of the 100 kHz field modulations was 0.1 mT. Lactate dehydrogenase (LDH) in the outflow was monitored to confirm the viability of the perfused liver. LDH in the outflow were below 10 IU/l throughout experiments.

Measurement of hydroxylamine form

At the end of each ESR experiment, the liver was perfused with KHB and removed to prepare liver homogenates. After homogenizing in ice-cold phosphate buffered saline, amounts of nitroxyl probes in liver homogenates was quantified before and after reoxidation with potassium ferricyanide to evaluate the amount of hydroxylamine form [11] using a X-band ESR. Amounts of nitroxyl probes in bile and effluents from liver *in situ* were also quantified with the same procedure.

Results

In this study, we investigated liver uptake of Carboxy PROXYL and quantified amounts of the nitroxyl and hydroxylamine forms, which is a major metabolite of the spin probe, in perfusate, liver homogenates or bile after rat liver perfusion *in situ*. ESR signal height of Carboxy PROXYL in the effluent (Figure (1c)) gradually increased and reached a steady state after introducing a Carboxy PROXYL containing buffer into *in situ* rat liver (Figure (2a)). The fractionated effluents were reoxidized with potassium ferricyanide [11] to quantify amount of hydroxylamine form of Carboxy PROXYL in the effluent (open rectangle in Figure (2a) represents ESR signal intensity after reoxidation with potassium ferricyanide). Hydroxylamine form of nitroxyl radical is reoxidized to nitroxyl form with potassium ferrycianide [11].

Figure (2a) shows total amount of Carboxy PROXYL in the effluent was ca. 60% of the influent at the steady state. Since significant amount of hydroxylamine of Carboxy PROXYL was detected in the liver homogenates and ESR signal of Carboxy PROXYL in the bile gradually increased during the perfusion, it was suggested that the liver uptakes Carboxy PROXYL *in situ*. To further investigate possible uptake of Carboxy PROXYL to liver tissue, liver perfusion with Carbamoyl PROXYL was also carried out. Carbamoyl PROXYL is another spin probe frequently used in *in vivo* ESR study [7,18,21,22] and has a larger octanol/water partition coefficient than Carboxy PROXYL (Table I). When Carbamoyl PROXYL was perfused into the *in situ* liver, all the spin probe was detected in the effluent

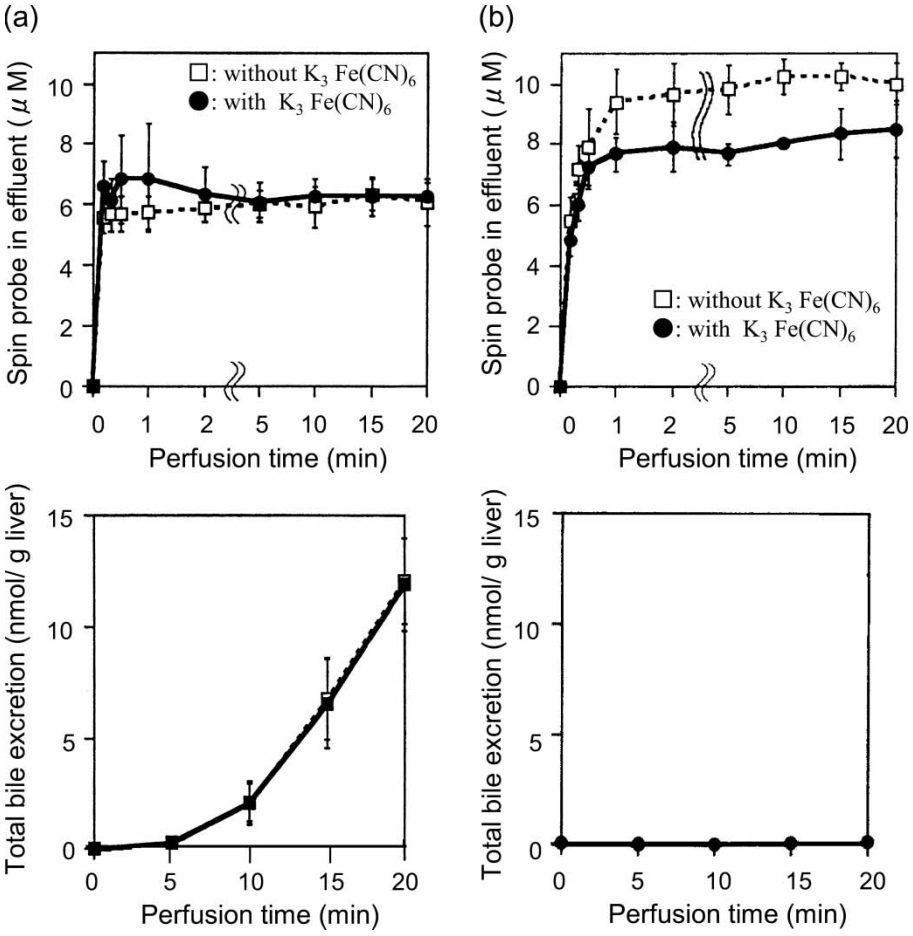


Figure 2. Time course of ESR signal intensity of (a) Carboxy- or (b) Carbamoyl PROXYL in the effluent (top) and in bile (bottom) after perfusion through rat liver *in situ*. In both figures, ESR spectra were measured without reoxidation (closed circle) or with reoxidation (open rectangle) by potassium ferricyanide as described in "Materials and methods" section. All values represent the mean with standard deviation.

either in nitroxyl or hydroxylamine form (Figure (2b)). Trace amount of the spin probe was detected in the bile. The difference in the perfusion profiles between Carboxy- and Carbamoyl PROXYL indicated that the disappearance of Carboxy PROXYL from the effluent was due to its transfer of Carboxy PROXYL to liver tissue. These results suggest that a specific transporting system may be responsible for the transfer of Carboxy PROXYL in rat liver *in situ*.

In situ liver perfusion with Carboxy PROXYL was then carried out for 20 min to evaluate *in situ* liver uptake of Carboxy PROXYL during the perfusion. During the perfusion, *in situ* rat liver with 10 μM of Carboxy PROXYL for 20 min, 442 ng/g liver of nitroxyl radicals was detected in the perfusate, in which 16 ng/g liver was in the hydroxylamine form, and 112 ng/g liver of Carboxy PROXYL was as the hydroxylamine form in the liver tissue (Table II) as hydroxylamine form. Small amount of Carboxy PROXYL (14 ng/g liver) were detected in the bile as hydroxylamine form. Carbamoyl PROXYL showed a different uptake profile. Seven hundred and ninety ng per gram liver of the spin probe was observed in the effluent, which is closed to the entire spin probe

Table II. Distribution of Carbamoyl- and Carboxy-PROXYL during liver perfusion.

		Carboxy PROXYL (nmol/g liver)	Carbamoyl PROXYL (nmol/g liver)
Perfusate	Nitroxyl form	426.1	639.1
	Hydroxylamine form	15.9	150.7
Liver homogenate	Hydroxylamine form	112.2	5.7
Bile	Hydroxylamine form	14.1	<0.1
Unknown		231.7	4.5

Hydroxylamine form was evaluated as the increases of signal intensity of the spin probe after reoxidation with potassium ferricyanide. Reoxidation process was carried out as described in "Materials and methods" section.

perfused and only 5.7 ng/g liver was detected in the homogenates. Since the amount of Carbamoyl PROXYL in the liver was smaller than that of Carboxy PROXYL while Carbamoyl PROXYL has larger partition coefficient than Carboxy PROXYL, above result supported the presence of active transporting system of Carboxy PROXYL in the rat liver *in situ*.

Carboxy PROXYL concentration in the effluent increased by additions of *p*-aminohippuric acid or penicillin G, typical competitive inhibitors of OATs (Figure (3)), indicating that Carboxy PROXYL uptake to rat liver *in situ* was competitively inhibited. As can be seen, inhibition of OATs by *p*-aminohippuric acid or penicillin G resulted in a concentration dependent increase of the spin probe in effluent. LDH in the effluents was not affected by additions of either *p*-aminohippuric acid or penicillin G, supporting that there was no cytotoxic effects by *p*-aminohippuric acid or penicillin G in this experiments that potentially affect on hepatic uptake of Carboxy PROXYL.

Discussion

Extensive studies have been carried out on distributions of nitroxyl spin probes *in vivo*, mostly using mice (for reviews, see Kocherginsky and Swartz [5]). Recent development of very low frequency ESR spectrometer using radio frequency under 1 GHz [3,18] or 300 MHz [4] enables us to measure free radicals and to assess pharmacodynamics of nitroxyl radicals in intact mice or rats.

An uptake of an ESR contrast reagent, Carboxy PROXYL, to rat liver *in situ* was of interest, since the uptake could not be explained with passive transport of Carboxy PROXYL to liver. Carboxy PROXYL is an anionic nitroxyl spin probe and therefore regarded to be poorly transfered to tissues based on data using mice models [18], and no active transporting system of nitroxyl spin probes was reported *in vivo* or *in situ*. We showed in this study that Carboxy PROXYL was taken up, to rat liver *in situ* through OATs, by demonstrating competitive suppression of the uptake of nitroxyl spin probe by *p*-aminohippuric acid or penicillin G.

In vivo electron spin resonance spectroscopy/imaging (ESR/ESRI) is a unique technique that measure free radicals *in vivo* non-invasively. *In vivo* ESR/ESRI technique usually requires an injection of a spin probe that is a paramagnetic reporter molecule, since endogenous radicals are in low concentrations to be measured directly. Current results would be useful in the choice of spin probes to explore the possibility of *in vivo* ESR/ESRI as a modality to measure and visualize *in vivo* OAT activity.

Several publications reported fluorescence labeling as a technique to visualize the OATs activity. [1,8] Fluorescence technique would be limited in its application in OAT or Oatp study to *in vitro*, *in situ* experiments, while *in vivo* ESR technique may be applied to whole mice or rats non-invasively. One of the other possible modalities for non-invasive OATs imaging is magnetic resonance imaging (MRI). Van Montfoort et al. [20] reported that a hepatobiliary MRI contrast agent was taken up by organic anion transporting polypeptides *in vitro*. MRI technique has not been applied to visualize OATs *in vivo* as far as we know, and a comparison between two imaging modalities has not been done yet.

In current study, we employed non-specific competitive inhibitors for OATs, *p*-aminohippuric acid and penicillin G [12] to examine mechanisms for Carboxy PROXYL uptakes by rat liver *in situ*. It is also necessary to explore the varieties of ESR spin probe for *in vivo* ESRI to find or design an optimal spin probe. One reason for it is OAT transfers structurally unrelated compounds [9,17], which makes it difficult to find an optimal spin probe from their structures. A stable spin probe in biological system is also preferable for an imaging transfer through OAT *in vivo*. Carboxy PROXYL has been used for analyzing free radical reactions or redox status *in vivo* [16,21]

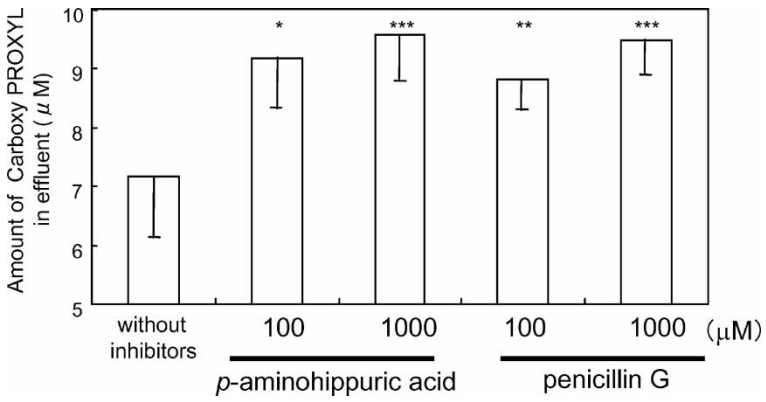


Figure 3. Effect of competitive inhibitors of organic anion transporter on recovery (unchanged form) of Carboxy PROXYL in the effluent. All values represent mean ± SD of 6–7 experiments. Symbols represent statistical differences: * $P < 0.01$; ** $P < 0.005$ and *** $P < 0.001$.

and gradually reacts with reducing systems or free radicals in biological systems [5]. Though pyrrolidine type of nitroxyl spin probes, to which Carboxy PROXYL belongs, are more stable than piperidine nitroxyl radicals in biological systems, the usage of Carboxy PROXYL could be limited as a contrast reagent for imaging OAT *in vivo*. OATs are found in many tissues, and the distribution of OATs is different among tissues and species [12,19].

During 20 min of perfusion, 112.2 ng/g liver of Carboxy PROXYL was detected as hydroxylamine in the liver homogenates (Table II), which is completely different from that of Carbamoyl PROXYL. Kroll et al. [6] reported that secondary amine was formed as well as hydroxylamine from TEMPO derivatives in human keratinocytes by using HPLC. We also found that secondary amine was formed from nitroxyl radicals in the presence of electron donor (unpublished data). Undetected portion of Carboxy PROXYL might include therefore its corresponding secondary amine, which is not reoxidized to Carboxy PROXYL form with potassium ferricyanide. The processes should proceed after Carboxy PROXYL transport from perfusate to liver tissue.

In summary, we reported, for the first time as far as we know, that a nitroxyl ESR contrast reagent is actively transported through organic anion transporter families to rat liver *in situ*. The current result would be a first step for imaging of OAT *in vivo* with ESR technique.

Acknowledgements

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