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EVIDENCE OF *IN VIVO* FREE RADICAL GENERATION BY SPIN TRAPPING WITH α-PHENYL N-*TERT*-BUTYL NITRONE DURING ISCHEMIA/REPERFUSION IN RABBIT KIDNEYS

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By using α-phenyl N-tert-butyl nitrone (PBN) as spin trap molecule and the electron paramagnetic resonance (EPR) technique, we obtained the first direct evidence of *in vivo* intervention of free radicals during an ischemia (50 minutes) reperfusion phenomenon in kidney of an intact rabbit.

An EPR signal (triplet of doublets) characterized by coupling constants a_N = 14.75-15 G and a_β^H = 2.5-3 G was detected in blood samples. The signal was consistent with a nitroxyl-radical adduct resulting from the spin trapping by PBN of either oxygen-or carbon-centered radicals. Control experiments indicated that the EPR signal was not due to a toxic effect of the spin trap molecule.

KEY WORDS: Ischemia, free radicals, spin trapping, EPR technique, kidney.

INTRODUCTION

In recent literature, the post-ischemic reperfusion injury is often attributed to the action of free radicals, generally oxygen reactive metabolites.[1] However most of the evidences supporting a role of free-radicals remain indirect.[2-9] In order to afford a direct evidence of the involvement of free radicals, the electron paramagnetic resonance (EPR) technique has been used, especially in *in vitro* models such as isolated rabbit[10] and rat[11-14] hearts subjected to global ischemia and reperfusion.

In contrast, few data were published about free radicals production during *in vivo* ischemia. After two inconclusive reports of Rao *et al.*[15] and of Arroyo *et al.*,[16] Bolli *et al.*[17] unequivocally demonstrated the production of free radicals by intracoronary infusion of the spin trap α-phenyl N-*tert*-butylnitrone (PBN) in the intact dog subjected to a coronary occlusion and subsequent reperfusion: stable adducts formed by reaction of spin trap molecules with free radicals were so detected in blood by the EPR technique.

Using the same method, we afford the first evidence of an *in vivo* production of free radicals in the intact rabbit kidney subjected to an ischemia-reperfusion phenomenon.

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MATERIAL AND METHODS

Adult New Zealand white rabbits (n = 5) weighing 3 kg were incubated, anaesthetized (fluothane, sufentanil) and paralysed with pancuronium. Central venous and arterial pressures and central temperature were monitorized. During all the study, animals were kept in normothermia (> 35.5°C) with a heating blanket. Infusions of colloid solutions (Haemacel) were used as needed to maintain the systolic blood pressure close to 60–70 mm Hg until the end of the blood sample collection. Arterial pH, partial oxygen pressure (pO_2),pCO_2 were maintained in the normal range by adjusting the ventilatory parameters. After a mid-line laparotomy, the right kidney was removed. Thereafter, the superior mesenteric artery (SMA), the aorta (AO), the inferior vena cava (IVC) and the left renal pedicle (LRP) were dissected with ligature of all extra-renal branches. Two catheters were introduced through the AO and the IVC with their tips just below the LRP. Then the aorta was cross-clamped between SMA and LRP for 50 minutes. A first 5 minutes-aortic infusion of PBN (20 mg/ml, 1.5 ml/min) freshly dissolved in a mixture of 80% normal saline and 20% water started with the aortic occlusion in order to permit PBN to specifically diffuse into the kidney. PBN infusion (20 mg/ml) was started again 5 minutes prior to reperfusion at the rate of 1 ml/min and continued during the collection of blood samples. At reperfusion, the SMA was ligated to ensure strict left renal perfusion by PBN and IVC was ligated just above the left renal vein to avoid retrograde contamination by the systemic blood. Through the IVC catheter, 8 blood samples (8 ml) were successively drawn over a period of 6 ± 2 minutes by means of syringes containing 200 UI of heparin. After immediate centrifugation, plasma samples were frozen (− 196°C) in liquid nitrogen until lipids extraction according to the method of Folch *et al.*[18] After evaporation of the chloroform layer until dryness, lipid residues were pooled in 300 µl of chloroform immediately transferred into a quartz flat cell which was then placed in the sample cavity of a Varian E109 EPR spectrometer equipped with a 100 kHz modulation. All the spectra were recorded at 25°C with the following settings: field 3425 G, microwave power 20 mW, modulation amplitude 1 G, time constant 1 sec, scan range 100 G, and scan time 8 minutes.

In one additional experiment (ischemia period of 60 minutes), EPR signal was searched in the pooled lipidic residue of the four first blood samples (0–3 minutes period following reperfusion). Similar operation was made for the blood samples taken between the 3–6 minutes period after reperfusion.

In three control experiments to ensure that the EPR signal cannot be attributed to a nonspecific effect of PBN, we infused the spin trap (20 mg/ml, 1.5 ml/min) through the kidney non subjected to occlusion. Eight blood samples (8 ml) were successively drawn over a period of ten minutes.

RESULTS

As shown on Figure 1a, a renal ischemia of 50 minutes provokes the apparition in the blood of an EPR signal characterized by a triplet of doublets, with the coupling constants a_N = 14.75–15 G and a_β^H = 2.5–3 G and with asymmetry of the central doublets. With a gain of 5 × 10^5, the total height of the second doublet (taken as arbitrary unit) is 140 ± 40 mm (n = 5). The signal is consistent with a nitroxyl-radi-

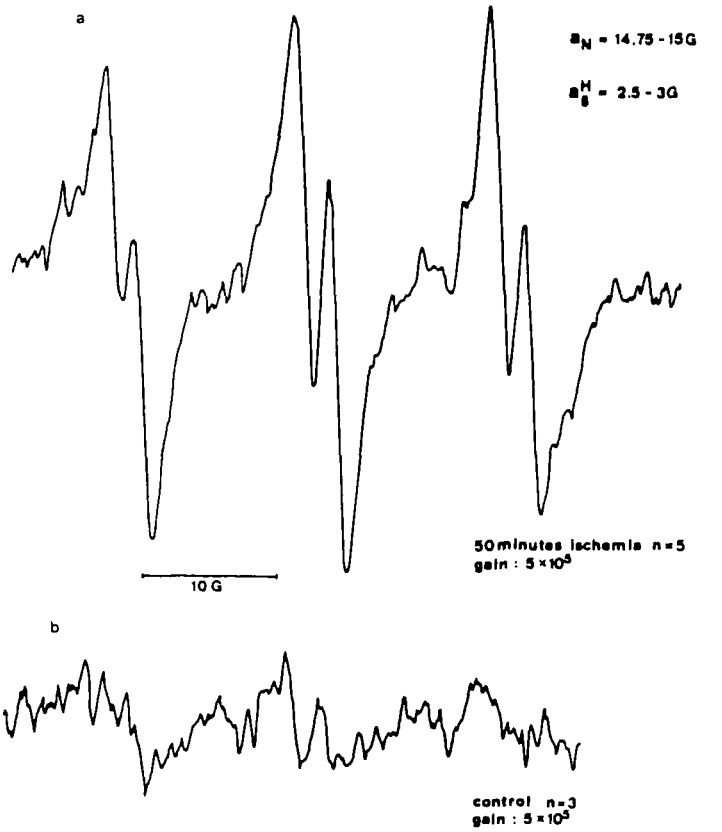

FIGURE 1 a) Typical EPR spectrum of PBN radical adducts detected in the effluent (8 plasma samples) of rabbit kidney subjected to an ischemia (50 minutes)-reperfusion phenomenon (a_N = 14.75–15G, a_β^H = 2.5–3G; gain 5 × 10^5). Registration conditions are given in Material and Methods. b) EPR signal from 8 plasma samples obtained after PBN infusion (similar quantity as in a) through a kidney non subjected to occlusion (gain 5 × 10^5).

cal adduct resulting from the spin trapping by PBN of either oxygen-or carbon-centered radicals.

In contrast, no EPR signal is detected in the blood when the spin trap is infused through a kidney non subjected to occlusion (n = 3, Figure 1b).

Figure 2 shows that a weak and badly defined signal can be observed in the four blood samples collected during the three initial minutes after reperfusion (a). On the other hand, the intensity of the signal is strongly increased in the four samples taken for the 3–6 minutes period after reperfusion (b). The EPR signal is well defined with coupling constants a_N = 15G and a_β^H = 3G (gain 1 × 10^6).

DISCUSSION

Indirect proof of *in vivo* free radicals production in kidneys after an ischemic-reper-

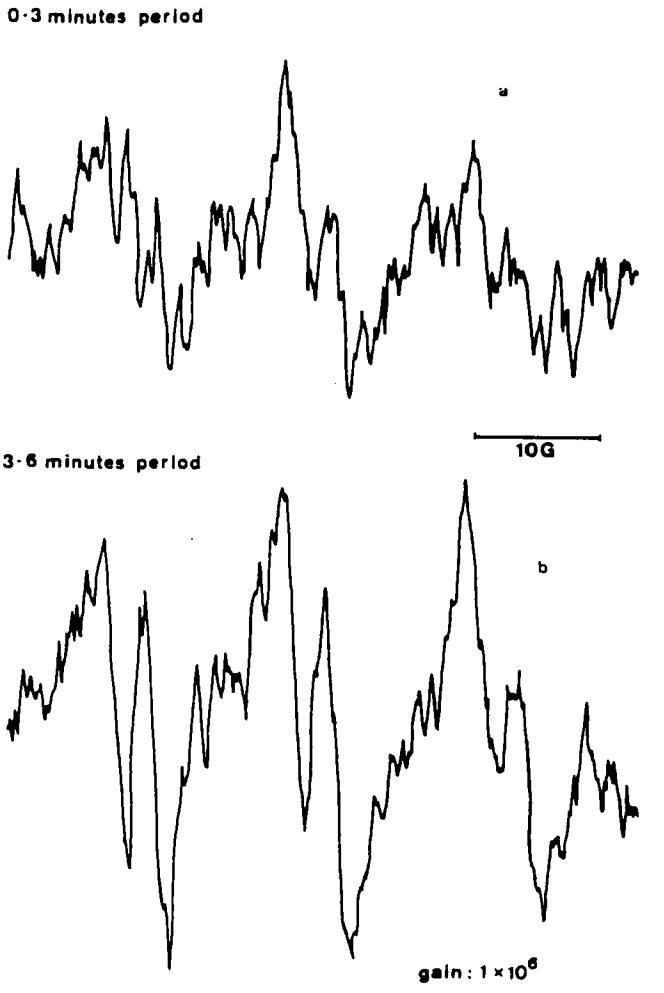

FIGURE 2 EPR signal corresponding to the 0–3 minutes period (part a; 4 blood samples) and to the 3–6 minutes period (part b; 4 blood samples) following kidney reperfusion (gain 1 × 10^6).

fusion phenomenon have been previously given in the literature.[19,20,2,4,6,21,22] In this brief report, we afford supplementary support to these preliminary data by direct evidence of free radicals formation using α-phenyl N-*tert*-butyl nitrone (PBN) as spin trap molecule and electron paramagnetic resonance (EPR) technique.

The solubility of these spin adducts in an apolar phase (chloroform) and the similarity of the signals with those previously observed after *in vivo* γ-irradiation[23] or CCl_4[24] administration of animals treated with PBN, strongly suggest that these radicals may derive from the attack of the membrane polyunsaturated fatty acids (RH) by oxygenated free radicals generated during the ischemic-reperfusion phenomenon. These lipidic radicals can be either carbon-centered such as R· radical or oxygen-centered such as alkoxyl (RO·) and peroxyl (ROO·) radicals.

Our a_N values (14.75–15 G) in chloroform as solvant are larger than those given for oxygen-centered radical adducts of PBN (13.6 G) while the a_β^H values (2.5–3 G) are smaller than those generally observed with a carbon-centered radical adduct of PBN (3.25–3.5 G).[16] This indicates tha the EPR signal observed is probably due to a mixture of both centered radicals as suggested by Bolli *et al.*[17]

The absence of signal in the renal venous effluent of the control group unequivocally contradicts the possibility of a nonspecific toxic effect of PBN and demonstrates that the EPR signal is well related to free radicals production.

In contrast with Bolli's study,[17] we were unable to establish a time course of PBN adduct production. In our experimental conditions, it is only when all lipid residues are pooled in 300 μl chloroform that paramagnetic species can be detected. This discrepancy could be explained by dissimilarities in the sensitivity of the EPR apparatus we used, but also in the tested organ (kidney versus heart). Nevertheless, in one recent experiment, it has been possible to evidence that the production of free radicals in blood is more important during the 3–6 minutes period after reperfusion than during the three initial minutes (Figure 2). This is in good agreement with the demonstration of a burst in PBN adduct production peaked at 2–4 minutes after the reperfusion in the heart model.[17]

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