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OXIDATIVE DNA DAMAGE AFTER TRANSPLANTATION OF THE LIVER AND SMALL INTESTINE IN PIGS

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Oxidative damage is thought to play an important role in ischemia/reperfusion injury, including the outcome of transplantation of the liver and intestine. We have investigated oxidative DNA damage after combined transplantation of the liver and small intestine in 5 pigs. DNA damage was estimated from the urinary excretion of the repair product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). In the first 1–3 hr after reperfusion of the grafts, 8-oxodG excretion was increased 2.9-fold (1.7–4.1; 95% confidence intervals; $P<0.05$). A control experiment included sham surgery with clamping of the suprarenal inferior caval vein in 2 pigs during steady state infusion of 8-oxodG. While the caval vein was clamped, the urinary excretion of 8-oxodG was almost blocked, whereas after removal of the clamp, the excretion returned to and did not exceed the preclamp levels. In a separate experiment with 2 pigs, the elimination of injected 8-oxodG was shown to adhere to first-order kinetics with a clearance and a terminal elimination half-life of approximately 4 ml min^{-1} kg^{-1} and 2½ hr, respectively. The injected dose was completely excreted into the urine within 4 hr. It is concluded that substantial oxidative damage to DNA results from reperfusion of transplanted small intestine and liver in pigs, as estimated from the readily excreted repair product 8-oxodG.

Reperfusion of ischemic tissue, including liver and intestine, leads to generation of reactive oxygen species through a variety of mechanisms (*1*, *2*). Indeed, free radicals have been directly demonstrated by electron spin resonance in reperfused ischemic organs, including liver and intestine (*3–5*). Accordingly, reactive oxygen species appear to be pathogenically important in reperfusion injury of ischemic organs such as liver and intestine, in particular in relation to transplantation (*2*, *4*).

So far, most studies of reoxygenation injury have focused on cellular and organ survival and function, morphology, and lipid peroxidation in terms of thiobarbituric reactive substances or aldehydes (*6*, *7*). Oxidative damage to specific molecules has rarely been identified. In relation to cancer and aging, DNA has been recognized as an important target for oxidative damage (*8*). In fact, signs of DNA damage in terms of nick translation and thymidine incorporation have been demonstrated after reperfusion of ischemic rat liver (*9*). The most abundant oxidative damage to DNA involves the C-8 position of guanine. In normal tissue DNA, 1 to 25 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)* are found per 10^5 deoxyguanosine, and higher levels are found with advanced age, in malignant tumors, and after treatment with ionizing radiation or chemical carcinogens (*10-13*). In vivo, such DNA

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* Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

damage is continuously repaired and the excretion of one resulting product, 8-oxodG, into the urine, thus reflects the rate of damage (*10*, *13–16*).

In the present study, we investigated oxidative DNA damage estimated by the urinary excretion of 8-oxodG in relation to orthotopic transplantation of the small intestine and liver in pigs. In addition, we studied the elimination kinetics of intravenously administered 8-oxodG.

MATERIALS AND METHODS

Animals. Ten female pigs (Danish Landrace; 12 weeks, 30–35 kg body weight), 5 donors and 5 recipients, were used for the experiments. Four other pigs were used for pharmacokinetic experiments. Animals were fasted for 18 hr before the operation but were allowed free access to water.

Anesthesia. The pigs were anesthetized with ketamine 7 mg kg^{-1} i.m. followed by thiopentone 8 mg/kg in an ear vein. After intubation, anesthesia was maintained with halothane (1–2%) in oxygen (100%) supplemented with fentanyl and diazepam as needed. Muscle relaxation was achieved with pancuronium. Dopamine 2–4 μg kg^{-1} min^{-1} was administered to facilitate renal function.

Donor operation. In brief, a laparotomy was performed through a midline incision. The liver and the small bowel were mobilized. The vessels supplying the pancreas and the colon were ligated and transsected. After heparinization, a catheter was inserted in the lower abdominal aorta. After cross-clamping the aorta just below the diaphragm, the combined liver-small bowel graft was perfused and cooled in situ with 1500–2000 ml of Ringer-lactate solution (4°C). A Carell patch containing the origin of the celiac axis and superior mesenteric artery was removed with the specimen. The graft was placed in an ice bath until reimplantation.

Recipient operation. A sibling to the donor pig was used as recipient. The recipient operation was started immediately after completion of the donor operation. After induction of anesthesia, catheters were placed in the transverse cervical artery, the jugular vein, and an ear vein for monitoring of arterial and venous pressures, drug administration, and blood sampling, respectively. A catheter was inserted in the bladder for urine sampling.

A laparotomy was performed through a midline incision. The liver and small bowel were resected. During the anhepatic phase, the portal vein flow was directed to the external jugular vein by a pump-driven, venovenous bypass that did not require heparinization. The graft was implanted by supra- and infrahepatic caval anastomosis. The portal vein of the recipient was anastomosed end-to-side to the donor portal vein with Prolene 5–0 sutures. After completion of this anastomosis, the liver was reperfused with portal blood. The donor superior mesenteric artery was anastomosed to a recipient jejunal artery. The donor celiac trunk was anastomosed end-to-side to the recipient common hepatic artery. After arterial reperfusion, the donor common bile duct was anastomosed to the recipient common bile duct.

Immediately before venous reperfusion of the liver and arterial reperfusion of the small bowel, the organs were perfused with temperate saline in order to flush out toxic metabolic intravascular substances. The mean duration of the anhepatic phase was 57 min (range 46–69). The mean cold ischemic period of the organ graft was 201 min (range 181–218).

Postoperatively, the recipient pigs remained anesthetized for 2 to 3 hr for urine sampling while the circulation was stable, the diuresis was maintained, and there were no signs of bleeding. Thereafter, the pigs were killed with a bolus of intravenous KCl.

8-OxodG pharmacokinetics. The elimination pharmacokinetics of 8-oxodG was investigated by administration of the compound yielding plasma and urine concentrations 100–1000 times the spontaneous levels. A solution containing 8-oxodG 700 μM as final concentration was generated from a solution initially containing 2′-deoxyguanosine 2.5 mM, EDTA 6.5 mM, ascorbic acid 14 mM, and $FeSO_4$ 1.3 mM by bubbling with 100% oxygen for 3 hr at 37°C (*17*). The solution was calibrated against pure compound kindly supplied by Dr. David W. Potter, Rohm and Haas Co. (Spring House, PA), and Dr. Per Leanderson, Department of Occupational Medicine, Linköping Hospital (Sweden).

Two anesthetized pigs received 4 μmol of 8-oxodG as an intravenous bolus over 10 min. Plasma was sampled before and 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min later. Urine was collected in 30-min intervals.

Clearance was calculated from the ratio of the dose and the area under the concentration-time curve estimated according to the trapezoidal rule. The elimination half-life, $t_{½\beta}$, was determined from the elimination rate constant estimated by linear regression of the terminal part of the log concentration time curve. The apparent volume of distribution (V_β) was determined as the clearance divided by the elimination rate constant. The plasma concentration-time data were fitted to a 2-compartment model by means of nonlinear regression weighted by the reciprocal of the square value.

Two anesthetized pigs received a bolus dose of 8-oxodG 30 nmol kg^{-1} i.v. over 10 min followed by an infusion of 9 nmol kg^{-1} hr^{-1} estimated from the single dose clearance and V_β to yield a plasma concentration of approximately 40 nM. Plasma was sampled before and at 15-min intervals for 6 hr and urine was collected in 30-min intervals. Steady state clearance was calculated as the ratio between the infusion rate and the plasma concentration. Sham surgery was performed through a midline laparotomy. Starting after 2- or 3½-hr infusion of 8-oxodG in each pig, the inferior caval vein was clamped immediately above the renal veins for 1 hr in order to expose the kidneys to almost the same physiologic conditions as during the anhepatic phase.

8-OxodG analysis. Urine samples from the transplantation studies were analyzed for 8-oxodG by a previously published three-dimensional HPLC method with electrochemical detection (*15*). Plasma from the pharmacokinetic studies was precipitated with an equal volume of perchloric acid 2 N. A 10-μl aliquot of the 4000 g supernatant was injected on a Nucleosil ODS 5 μ 15-cm column eluted with methanol/acetonitrile/phosphate buffer (pH 2.5) (3/2/95, v/v/v). The effluent was monitored by a Coulochem II electrochemical detector (ESA, Inc., Bedford, MA) with a 5010 porous graphite analytical cell run in the oxidation mode (E_1=120 mV, E_2=280 mV). Urine from the pharmacokinetic studies was injected after 10-fold dilution with water. Calibration graphs constructed from addition of pure compound to blank samples were linear in the relevant ranges. The intra- and interassay coefficient of variation for determination of 8-oxodG in urine is 8% and 10%, respectively.

Statistics. The rates of 8-oxodG excretion before and after reperfusion were compared by means of the paired *t* test. For the relative change, 95% confidence intervals were calculated.

RESULTS

Effect of reperfusion on 8-oxodG excretion. The basal level of urinary 8-oxodG excretion in the 5 pigs was 0.96 (0.65–1.46) nmol hr^{-1} (median with range). In the first 2 hr after reperfusion, the excretion increased 2.9-fold (1.68–4.09; 95% confidence interval) to 2.37 (1.21–5.15) nmol hr^{-1} ($P<0.05$) on average (Fig. 1). During the observation period, this increase reached an apparent maximum in 3 of the pigs.

8-oxodG pharmacokinetics. Before injection of 8-oxodG, the plasma concentration was 1–2 nM. After intravenous administration, the plasma decay of injected 8-oxodG complied with a 2-compartment model with a rapid distribution phase and a slow elimination phase (Fig. 2). The elimination appeared to adhere to first-order kinetics and a clearance of approximately 4 ml min^{-1} kg^{-1}. V_β and $t_{½\beta}$ were approximately 1 L kg^{-1} and 2½ hr, respectively (Table 1). Within 4 hr, vir-

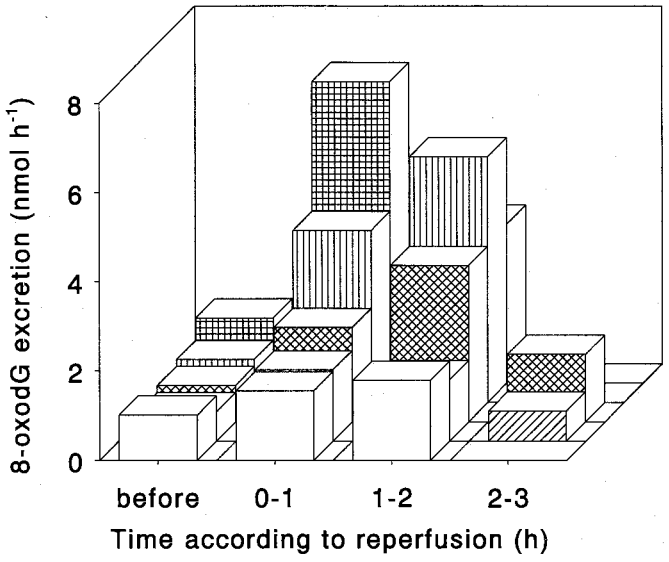

FIGURE 1. Individual urinary excretion rates of 8-oxodG before and after reperfusion in relation to transplantation of the liver and small intestine in 5 pigs. Each bar signature represents 1 pig.

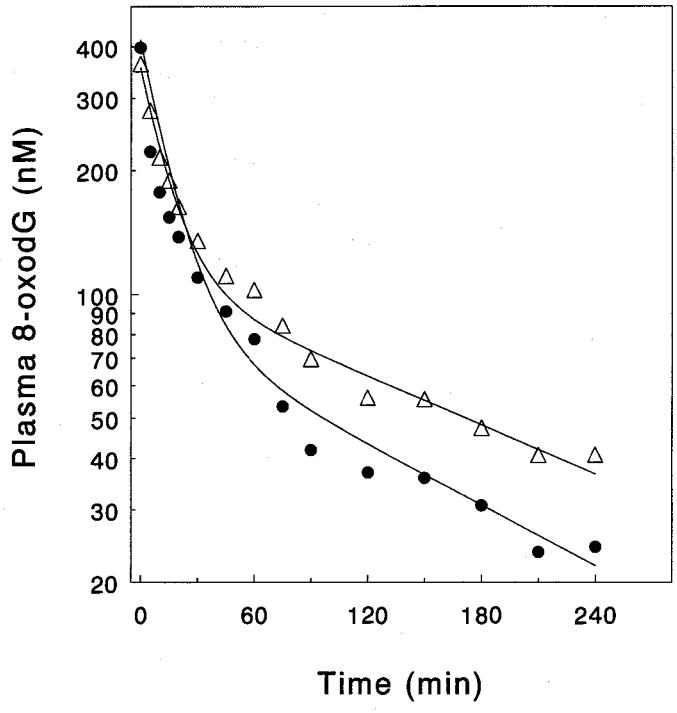

FIGURE 2. Plasma concentration-time curve after intravenous injection of 4 μmol of 8-oxodG over 10 min in 2 pigs. The lines represent the 2-compartment model equation derived from nonlinear regression.

tually the complete dose was excreted into the urine (Fig. 3).

Steady state kinetics of 8-oxodG were achieved by intravenous infusion (Fig. 4). The clearance calculated from the infusion rate and the steady state concentration was approximately 3.3 ml min^{-1} kg^{-1} (Table 1). Moreover, the urinary excretion rate corresponded to the infusion rate (Fig. 4). After clamping of the caval vein, the steady state plasma concentrations increased by 25–30%, reversible in one ping but not in the other. The caval clamp caused an almost complete block of the urinary excretion of 8-oxodG. This block lasted approximately an hour and was reversible but not followed by an increased excretion rate.

TABLE 1. Pharmacokinetic parameters of 8-oxodG in pigs

Pig	Single dose		Steady state	
	1	2	3	4
Clearance (ml min^{-1} kg^{-1})	4.8	3.7	3.2	3.4
Half-life (hr)	2.55	2.73	–	–
V_β (L kg^{-1})	1.04	0.87	–	–

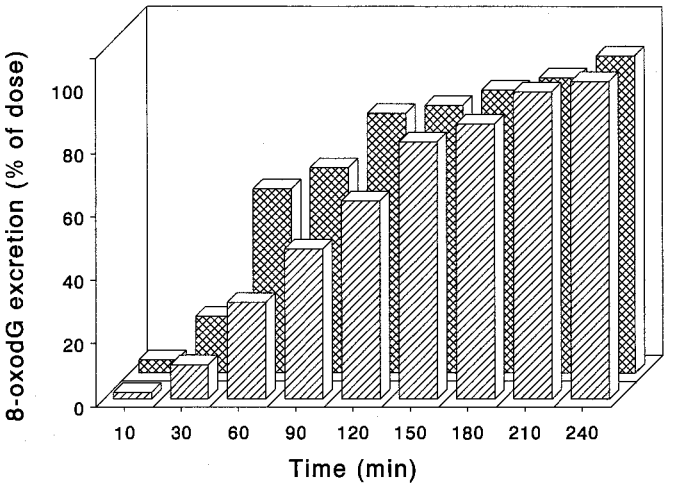

FIGURE 3. Cumulated urinary excretion of 4 μmol of intravenously injected 8-oxodG in 2 pigs. Each bar signature represents 1 pig.

DISCUSSION

The basal 8-oxodG excretion of approximately 1 nmol hr^{-1} in the present pigs corresponds to an average hourly repair of 25 oxidatively damaged guanine bases in the DNA of each cell in their body, assuming a total of 2.5×10^{13} cells. Thus, the 3-fold increase demonstrated in the first hours after combined transplantation of the liver and small intestine indicates substantial oxidative damage to DNA of the transplanted organs upon reperfusion.

Injected 8-oxodG was rapidly and completely excreted into the urine of pigs. In a separate experiment with sham surgery including a caval clamp, these procedures were shown to reduce the excretion temporarily. Previously, injected 8-oxodG has been shown to be readily excreted unchanged into the urine in rats, whereas oxidation of dG during excretion or ingested 8-oxodG from the diet does not contribute to the excretion (*14*, *18*). Accordingly, the present increase in 8-oxodG excretion after transplantation of the liver and intestine reflected an even more increased rate of deoxyguanosine oxidation in DNA or in the cellular pools of nucleosides and nucleotides. If not hydrolyzed by a specific enzyme (*19*), the latter may be incorporated into DNA and represent a mutation risk (*20*, *21*). The increase in 8-oxodG excretion within 1–2 hr after reperfusion seems too early to be a result of digestion of damaged DNA from dead cells and turnover of mitochondria. Besides 8-oxodG, the repair of this lesion in DNA may also result in the corresponding free base,

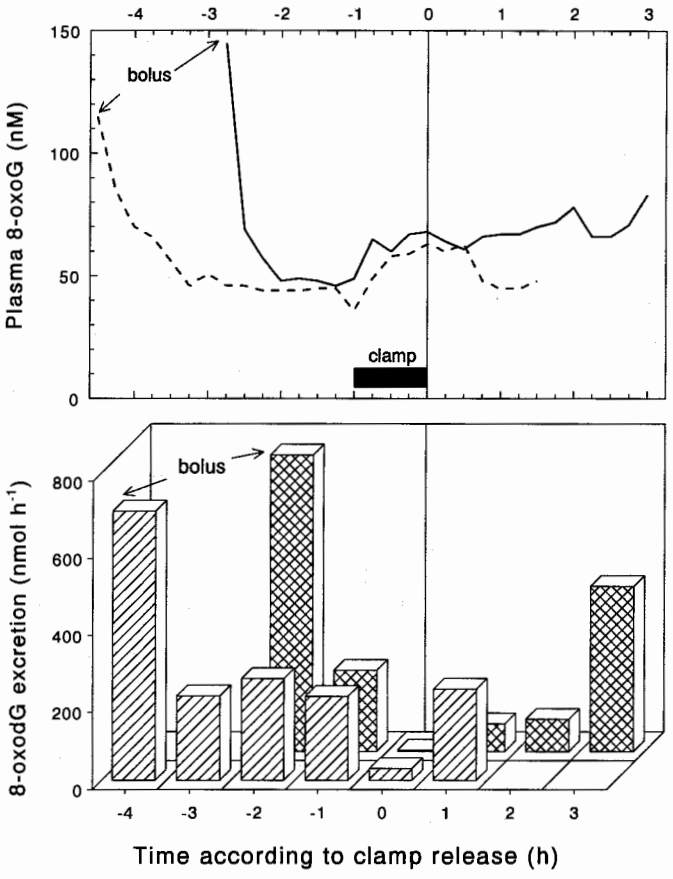

FIGURE 4. Individual steady state plasma concentration-time curve (top) and urinary excretion rate (bottom) during constant infusion of 8-oxodG after a bolus dose in 2 pigs before and after sham surgery involving clamping of the caval vein for 1 hr. The broken line (top) corresponds with the striped bars (bottom) for 1 pig, and the solid line (top) corresponds with the cross-hatched bars (bottom) for the other pig.

8-oxoguanine, and the relative importance of these alternative products is still undetermined (*18*). Moreover, although the most abundant, 8-oxodG formation is only one of a wide variety of oxidative damages to DNA bases and sugar residues and nucleotides (*11, 12, 22*). Thus, reperfusion injury to transplanted organs may include more substantial DNA damage than indicated by the increased 8-oxodG excretion. A concomitantly increased rate of mitosis due to cell killing implies a risk of failing DNA repair with large numbers of mutations and in turn the possibility of cancer (*8*), although an increased risk in transplanted organs has not been demonstrated so far. However, damage to DNA could be involved in graft dysfunction. The excretion of 8-oxodG may provide a valuable tool for monitoring oxidative reperfusion injury and its possible reduction or prevention by antioxidants and other measures.

Reperfusion injury involving cellular and organ survival, morphology, and lipid peroxidation has been demonstrated in many studies (*1, 2*). Indirect evidence of DNA damage has been provided by increased nick translation and thymidine incorporation after reperfusion of ischemic rat liver (*9*). However, the present data represent the first specific evidence of DNA base oxidation in relation to reperfusion of transplanted organs. Similarly, chemically induced oxidative stress to the liver or kidney in vivo includes cytotoxicity and lipid peroxidation as well as 8-oxodG formation in DNA (*23–25*). Moreover, lipid peroxidation products per se have been shown to induce 8-oxodG in isolated DNA (*26*).

Reactive oxygen species may be generated after reperfusion by formation of superoxide from xanthine oxidase catalyzed metabolism of accumulated hypoxanthine, dysfunction of the mitochondrial electron transport chain (*27*), and oxidative bursts from activated leukocytes (*2*). Further, hydrogen peroxide from dismutated superoxide may be a source of the most reactive oxygen species, hydroxyl radicals and hypochlorite, catalyzed by transition metals (*6, 28*) and myeloperoxidase from granulocytes, respectively (*7, 29–31*). The DNA damage indicated by the present data may be related to ROS from several of these mechanisms. However, whereas hydroxyl radicals generated from, for example, iron-catalyzed Fenton chemistry or ionizing radiation induce large quantities of 8-oxodG in DNA superoxide appears to lack this capacity (*32, 33*). Under normal circumstances, one of the most important sources of DNA damage may be ROS leaking from mitochondrial respiration (*34*), as suggested by correlations between the excretion of 8-oxodG and other similar biomarkers and the metabolic rate between species (*16, 35–37*) as well as in humans (*38*). Indeed, the basal excretion of 8-oxodG in young pigs was 2–3 times the corresponding human values, in keeping with a higher metabolic rate (*16*). In addition, activated leukocytes have been shown to induce 8-oxodG and other oxidative damage in the DNA of target cells (*39*). Which mechanism(s) is most relevant for oxidative DNA damage related to reperfusion may possibly be investigated by specific interventions, such as iron chelation (*6, 28*) or inhibition of leukocyte adherence (*30, 31*).

In replicating DNA, 8-oxodG leads to G-T transversions as well as other mutations and codon 12 activation of c-Ha-ras or K-ras oncogenes in mammalian systems (*20, 21, 40, 41*). In human tumors, G-T transversions are among the most frequent hot spot mutations in the p53 tumor suppressor gene (*42*). Reports of exponential accumulation of 8-oxodG and a correlation with deletions in mitochondrial DNA from human heart muscle indicate a role of oxidative DNA damage from the respiratory chain in the muscle weakness associated with aging (*43*).

This study demonstrated substantial oxidative damage to DNA from reperfusion of the transplanted pig liver and intestine, as estimated from the readily excreted repair product 8-oxodG. This excretion may be a valuable monitoring tool in intervention studies.

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