K⁺-Linked Release of Oxidized Glutathione Induced by *tert*-Butyl Hydroperoxide in Perfused Rat Liver Is Independent of Lipid Peroxidation and Cell Death

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ABSTRACT—The *tert*-butyl hydroperoxide (BHP)-induced release of oxidized glutathione (GSSG) and K⁺ was studied in relation to lipid peroxidation and cell death using isolated perfused rat livers. Infusion of BHP into the perfused liver resulted in an early and simultaneous release of GSSG and K⁺ and a sustained release of thiobarbituric-acid-reactive substances (TBARS) into the effluent perfusate, which was followed by further pre necrotic leakage of K⁺ followed by lactic dehydrogenase (LDH). These actions of BHP were not significantly affected by cutting or ligating the bile duct, and they were potentiated by omitting Ca²⁺ from the perfusion medium. Co-infusion of desferrioxamine, propyl gallate and diethyldithiocarbamate suppressed TBARS release as well as the later leakage of K⁺ and LDH. Desferrioxamine was also effective under Ca²⁺-free conditions. *N,N'*-diphenyl-*p*-phenylenediamine inhibited TBARS release, but it was not protective against cell death, although there was some delay. The action of dithiothreitol was only moderate. On the other hand, leakage of TBARS, K⁺ (pre necrotic) and LDH was enhanced by cysteamine and β -mercaptoethanol and most markedly enhanced by ferrous iron. However, none of these agents markedly affected the early release of GSSG and K⁺. These observations, which support our previous findings, suggest that the early and coupled sinusoidal efflux of GSSG and K⁺ caused by BHP is independent of lipid peroxidation and cell death and that they represent a physiological mechanism of GSSG release. The results also suggest that lipid peroxidation is not the sole cause of BHP-induced cell death.

Keywords: Perfused liver, *tert*-Butyl hydroperoxide, GSSG and K⁺ release, Iron chelator, Antioxidant

tert-Butyl hydroperoxide (BHP) is often used as a tool with which to study mechanisms of oxidative damage in various tissues such as the eye lens (1), red blood cells (2), liver (3–7) and heart (8, 9). This compound is detoxified to *t*-butyl alcohol by glutathione peroxidase and accompanies the oxidation of reduced glutathione (GSH) (3, 4). In the liver, the intracellularly accumulated oxidized glutathione (GSSG) is released into the sinusoidal space (3–5) as well as into the bile (5–7), resulting in a decrease of cellular glutathione levels. This disturbance in the redox balance of the cellular glutathione state and an efflux of GSSG is generally considered to be an index of oxidative stress (10). On the other hand, BHP induces lipid peroxidation in animals (11), isolated livers (12, 13) and cultured hepatocytes (14, 15). Hydroperoxides also induce microsomal lipid peroxidation in vitro (16, 17). The hepatocyte death induced by BHP may be primarily

due to lipid peroxidation, although lipid peroxidation-independent mechanisms such as mitochondrial damage cannot be ruled out (14, 18).

We recently reported that infusion of BHP into the isolated perfused liver produced an immediate, transient and coupled efflux of GSSG and K⁺ (GSSG/K⁺) into the effluent perfusate, which occurred even under conditions of suppressed lipid peroxidation in the presence of desferrioxamine, and is inhibited by high K⁺ perfusion medium, suggesting that the GSSG efflux is driven by a K⁺ gradient (19). In this study, we further examined the effects of various modifications of BHP toxicity on the efflux of GSSG and K⁺, to confirm that the coupled GSSG/K⁺ efflux is a physiological response to accumulated intracellular GSSG rather than a result of a pre necrotic permeability change in the hepatocyte plasma membranes due to lipid peroxidation. The relationships between GSSG/K⁺ efflux, lipid peroxidation and cell death are also discussed.

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MATERIALS AND METHODS

Animals and chemicals

Male, SPF-grade, Sprague Dawley rats, 100–130 g in weight, were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka; and they housed in an air-conditioned animal room (temperature $24 \pm 1^\circ\text{C}$, humidity 50–60%). Sources of the chemicals were as follows: BHP (Katayama Chemical, Tokyo); GSH, GSSG, desferrioxamine and cysteamine (Sigma, St. Louis, MO, USA); *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), *n*-propyl gallate, sodium diethyldithiocarbamate trihydrate, dithiothreitol, ferrous ammonium sulfate and 1-chloro-2,4-dinitrobenzene (Wako Pure Chemicals, Osaka); NADPH and glutathione reductase (Oriental Yeast, Tokyo).

Liver perfusion

The livers were isolated from rats (170–180 g, given food and water ad libitum) between 9:30 and 12:00 according to the basic procedure except that the smaller

lobes were tied and removed (20). The bile duct was cut close to the liver, and the bile was free to drain. In some experiments, the bile duct was ligated. The liver was sustained in a vessel containing perfusion medium, which was placed in a small box warmed with 37°C -circulating water. Perfusion was performed with a non-recirculating and constant flow (25 ml/min) system using Krebs-Henseleit bicarbonate buffer (KHB, 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 and 5.6 mM glucose, saturated with 95% O_2 –5% CO_2 at 37°C). The effluent temperature was kept constant at about 35°C by using a thermister placed in the outlet perfusate. BHP, dissolved in KHB, was infused 30 min after cannulation of the portal vein at a rate of 0.25 ml/min (final concentration $100\text{ }\mu\text{M}$, regularly). The tested compounds were usually dissolved in distilled water and infused from 10 min before BHP infusion to the end of the experiment at a rate of 0.025–0.25 ml/min using an infusion pump, unless otherwise noted. DPPD and 1-chloro-2,4-dinitrobenzene were dissolved in dimethylsulfoxide (DMSO) and infused at a rate of 0.025 ml/min.

Fig. 1. The release of GSSG, K^+ and LDH into the effluent perfusate by BHP infusion in the perfused rat liver. The effect of bile duct ligation. A: Bile duct cut. B: Bile duct ligation. Infusion of 1 mM BHP was begun 30 min after cannulation. Each point represents the mean $\pm$ S.E.M. ($n=5$ for A and B).

Effluent monitoring

Oxygen and K⁺ concentrations of the effluent perfusate were monitored using an oxygen electrode (Clark type) and a K⁺ ion electrode (Orion, Tokyo) connected to the outlet of the perfusion system (20). The effluent perfusate was collected for 15 sec at appropriate intervals. The GSSG concentration in the perfusate was assayed by following the oxidation of NADPH at 340 nm after addition of glutathione reductase (4). When 1 mM dithiothreitol was infused, Tietze's method (21) was applied. *S*-(2,4-Dinitrophenyl) glutathione in the perfusate was measured using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹ at 340 nm (22). Thiobarbituric-acid-reactive substances (TBARS) and lactic dehydrogenase (LDH) activity in the perfusate, as indices of lipid peroxidation and cell death, respectively, were determined as described previously (20). TBARS values were expressed as malondialdehyde equivalents. Desferrioxamine, antioxidants and sulfhydryl compounds at the concentrations tested had no effect on the color production of TBARS.

RESULTS

Effects of bile duct ligation

As shown in Fig. 1A, when 1 mM BHP was infused into the regular isolated liver preparation in which the bile duct was cut, GSSG and K⁺ concentrations in the effluent perfusate increased immediately and simultaneously, then decreased rapidly. The effluent TBARS concentration also increased rapidly, followed by a greater pre-necrotic K⁺ leakage and then by LDH leakage. We previously demonstrated the dose-response relationship between 0.33–3 mM BHP (19). Bile duct ligation had no effect on these BHP-induced changes (Fig. 1B). There was also no effect with 0.33 mM BHP (data not shown). The validity of this procedure is supported by studies of 1-chloro-2,4-dinitrobenzene infusion, in which the glutathione conjugate *S*-(2,4-dinitrophenyl) glutathione, was mainly excreted into the bile, that is, experimentally into the drain, whereas bile duct ligation caused a marked increase of the metabolite in the perfusate (Fig. 2). It should also be noted that, during glutathione conjugate excretion, the K⁺ release was quite low. In the following experiments, the bile duct was cut.

Effects of Ca²⁺-omission and iron

Omission of Ca²⁺ from the perfusion medium increased BHP-induced GSSG/K⁺ release about two-fold, and it slightly enhanced TBARS release and LDH leakage (Fig. 3A), whereas increasing the perfusate Ca²⁺ concentration to 2.6 mM did not significantly affect BHP-induced changes observed with regular KHB containing 1.3 mM Ca²⁺ (data not shown).

Fig. 2. The effect of bile duct ligation on the release of the glutathione conjugate following infusion 1-chloro-2,4-dinitrobenzene (CDNB) in perfused rat liver. CDNB was infused for 3 min at the concentrations and times indicated. Effluent perfusate was connected to a flow cell and the concentration of *S*-(2,4-dinitrophenyl) glutathione (DNB-SG) was continuously monitored at 340 nm. Finally, the amount of the DNB-SG drained into the medium in which the liver was suspended was also determined. Typical data are shown.

Infusing the perfused liver with 0.1 mM ferrous ammonium sulfate for 8 min prior to BHP infusion markedly enhanced both TBARS release and LDH leakage (Fig. 3B, bottom; the scale on the ordinate is doubled). However, GSSG release did not increase but rather slightly decreased. K⁺ leaked in an early single peak, probably the initial K⁺ release coupled with GSSG release being superimposed by accelerated pre-necrotic K⁺ leakage (Fig. 3B, top). Pretreatment with ferric chloride was not effective.

Effects of desferrioxamine

As shown in Fig. 4A, 0.1 mM desferrioxamine co-infusion, in the absence of Fe²⁺ pre-infusion, almost completely suppressed the TBARS release, K⁺ leakage in the later phase and LDH leakage induced by 1 mM BHP throughout the experimental period. The liver was apparently normal at the end of perfusion. However, the initial GSSG/K⁺ release was not suppressed at all; and furthermore, this K⁺ release was compensated by the following

Fig. 3. The effects of Ca^{2+} omission (A) and ferrous iron (B) on the release of GSSG, K^+ and LDH into the effluent perfusate by BHP infusion in the perfused rat liver. The control is shown in Fig. 1A. A: Perfusion medium was changed to Ca^{2+} -free KHB after 20 min. B: Ferrous ammonium sulfate (0.1 mM) was infused from 15 to 23 min and washed out for 7 min, and then 1 mM BHP was infused. Each point represents the mean $\pm$ S.E.M. ($n=3$ for A and 4 for B).

uptake phase. Similar effects were induced in the liver perfused in the absence of Ca^{2+} (Fig. 4B).

Effects of some antioxidants

Co-infusion with 0.01 mM DPPD had no effect on the initial GSSG and K^+ release, although a slight re-uptake of K^+ occurred. TBARS release was considerably suppressed, but LDH leaked with some delay (Fig. 5A). No prevention of LDH leakage was observed with 30 mM DPPD (data not shown). DMSO (a solvent for DPPD, final concentration of 0.1%) had no significant effect on the control and BHP-infused livers (data not shown).

BHP-induced TBARS and LDH leakage were almost completely suppressed by *n*-propyl gallate (0.1 mM) with some decrease of GSSG and K^+ release (Fig. 5B). Under these conditions, K^+ values also continuously decreased in the control livers, which was due to a direct effect of *n*-propyl gallate on the K^+ electrode.

Diethyldithiocarbamate (0.1 mM) also effectively,

though not completely, suppressed TBARS release and LDH leakage, without affecting the initial GSSG and K^+ release (Fig. 5C).

Effects of sulfhydryl and other compounds

Dithiothreitol, a thiol-protective compound, had no effect at 0.1 mM. At 1 mM, it moderately reduced the BHP-induced parameter changes (Fig. 6A).

Cysteamine (0.2 mM) enhanced TBARS release and accelerated LDH leakage (Fig. 6B, bottom; the scale on the ordinate is doubled), while GSSG and K^+ release decreased slightly (Fig. 6A, top). A similar result was obtained with 0.2 mM β -mercaptoethanol (data not shown).

GSH (1 mM), GSH-monoethyl ester (1 mM), cysteine (0.2 mM), penicillamine (0.2 mM), and hydroxyl radical scavengers such as mannitol (20 mM), benzoic acid (0.1 mM) and thiourea (0.1 mM) had virtually no effect on the initial K^+ release and the peroxidative damage induced by BHP.

Fig. 4. The effects of desferrioxamine on the release of GSSG, K⁺ and LDH into the effluent perfusate by BHP infusion in the perfused rat liver in the presence (A) or absence (B) of Ca²⁺. Compare with Figs. 1A and 3A. In both A and B, 0.1 mM desferrioxamine was infused after 20 min and BHP was infused after 30 min. In B, the perfusion medium was changed to Ca²⁺-free KHB at 20 min. Each point represents the mean $\pm$ S.E.M. ($n=4$ for A and 3 for B).

DISCUSSION

The efflux of GSSG under oxidative stress

The infusion of BHP and other oxidants into isolated perfused liver causes GSSG efflux into the perfusate (3–5). We previously showed that this GSSG efflux accompanies the simultaneous release of K⁺, not only after an infusion with BHP but also with the other oxidants, hydrogen peroxide and diamide (19). The coupled release of GSSG/K⁺ by BHP was dose-dependent, minimal with glutathione-depleted livers, and inhibited by high K⁺ perfusate. Thus, it was suggested that the GSSG efflux is driven by a K⁺ gradient (19). The present studies of bile duct ligation further confirms that GSSG is released through sinusoidal plasma membranes of hepatocytes rather than via the paracellular pathway, that is, from bile to the sinusoidal space through canalicular junctions, and that the amount of GSSG excretion into the bile was quite low as compared with that of its sinusoidal release.

This is in marked contrast to the glutathione conjugate DNB-GS, which was mainly excreted into the bile. In addition, the DNB-GS release into the effluent perfusate is accompanied only by a minimal release of K⁺ even when its excretion into bile was averted to the sinusoidal side by bile duct ligation. This indicates a specific coupling of sinusoidal K⁺ efflux with GSSG release but not with glutathione conjugate release.

The present study further supports the notion that the early sinusoidal GSSG/K⁺ efflux following BHP infusion is not caused by lipid peroxidation-related plasma membrane permeability changes: Irrespective of the suppression or enhancement of the degree of lipid peroxidation exerted by various types of agents, none of them markedly affected the coupled efflux of GSSG/K⁺.

The mechanistic coupling of K⁺ efflux with GSSG release is supported by the concomitance of the release over time and the amounts under various conditions. However, an exact quantitative correlation of both types

Fig. 5. The effects of some antioxidants on the release of GSSG, K^+ and LDH into the effluent perfusate by BHP infusion in perfused rat liver. Compare with Fig. 1A. DPPD (0.01 mM), *n*-propyl gallate (0.1 mM) and diethylthiocarbamate (0.1 mM) in A, B and C, respectively, were infused at 20 min, and 1 mM BHP was infused at 30 min. Each point represents the mean $\pm$ S.E.M. (n = 4 for A, 3 for B and C).

Fig. 6. The effects of dithiothreitol and cysteamine on the release of GSSG, K⁺ and LDH into the effluent perfusate by BHP infusion in the perfused rat liver. Compare with Fig. 1A. Dithiothreitol (1 mM) and cysteamine (0.2 mM) in A and B, respectively, were infused at 20 min, and 1 mM BHP was infused at 30 min. Each point represents the mean $\pm$ S.E.M. ($n=3$ for A and B).

of release is difficult, since the K⁺ release was soon compensated by uptake of K⁺ as observed in the presence of desferrioxamine or after a short exposure (5 min) to BHP (19). This K⁺ re-uptake is accomplished by a Na⁺-K⁺ pump, since it was completely prevented by ouabain (19). In some cases, this K⁺ re-uptake phase cannot be identified due to overlapping with an early pre-necrotic K⁺ release, as was most typical with ferrous iron (Fig. 3B), in which the initial K⁺ release was completely superimposed by a markedly hastened pre-necrotic K⁺ leakage. Thus, the initial K⁺ efflux coupled with GSSG efflux in itself is a reversible process, which can be mechanistically dissociated from pre-necrotic K⁺ leakage. It is notable that Ca²⁺-free perfusion medium increased both K⁺ and GSSG release, suggesting that extracellular Ca²⁺ regulates the coupled release of GSSG/K⁺ by altering membrane permeability.

Hepatic GSSG efflux under oxidative stress is conducted via canalicular and sinusoidal plasma membranes. The former transport system into the bile is considered to be

an ATP-dependent active process (23–26). The erythrocytes (2, 27) and heart (9), in which no anatomical device for GSSG excretion exists in the plasma membranes, also reportedly release GSSG in response to BHP exposure by an ATP-dependent transport mechanism. Our previous and present studies suggest that sinusoidal GSSG efflux is driven by the energy of the K⁺ concentration gradient and that ATP is required indirectly to maintain intracellular K⁺ concentration via operation of a Na⁺-K⁺ pump. This process may operate when biliary transport is saturated, such as under the rather high concentrations of BHP used in the present study, and may be a physiologically adaptive process to rapidly remove the excess endogenous oxidant GSSG from the hepatocytes.

The mechanism of the co-transport remains to be clarified. A direct involvement of ATP in this transport system is being investigated. Oxidation of mixed disulfide formation with a protein thiol in plasma membranes by intracellularly accumulated GSSG could also lead to an increase in K⁺ permeability.

BHP-induced cell death

The importance of lipid peroxidation is well recognized as a mechanism of tissue injury under various types of oxidative stress, although their causal relationship is not always clear (28). Iron is critical for the initiation and propagation of lipid peroxidation (29), and BHP-induced lipid peroxidation is thought to be triggered by alkoxyl or peroxy radicals produced in the presence of iron.

To support this, lipid peroxidation as well as cell death induced by BHP *in vivo* (11), in perfused livers (12) and in cultured hepatocytes (30) is reportedly enhanced by iron and inhibited by the specific ferric iron chelator (31) desferrioxamine. We also confirmed this here.

The inefficacy of the antioxidant DPPD in preventing liver damage even under suppression of lipid peroxidation was in agreement with our previous histological study (13). With cultured hepatocytes, Masaki et al. (30) demonstrated that cell death caused by low concentrations of BHP is dependent upon lipid peroxidation, that is, prevented by DPPD, but at high concentrations, other mechanisms are also involved. However, the antioxidant, *n*-propyl gallate, and diethyldithiocarbamate prevented both lipid peroxidation and cell death. The difference may be due to the further ability of these latter agents to form inert metal complexes. Conversely, cysteamine and β -mercaptoethanol enhanced lipid peroxidation and augmented cell death, which could be due to the formation of active iron complexes (32).

The antioxidant action of DPPD, because of its lipophilic nature, may be mainly attributable to competitive scavenging of lipid radical intermediates within cellular membranes, whereas water-soluble iron chelators prevent lipid peroxidation in a more basic manner, namely, by inhibiting the production of *t*-butyl alkoxyl or peroxy radicals. These BHP-derived radicals not only initiate lipid peroxidation but may also interact with other cellular components, which is another possible cause of cell death. As a DPPD-unrelated mechanism of cell death, mitochondrial damage has been proposed (33).

Although Ca^{2+} has been proposed as a toxic mediator of cell death (34), entry of extracellular Ca^{2+} may not be a critical requirement in BHP-induced cell death, since Ca^{2+} -omission rather enhanced lipid peroxidation and cell death. Sakaida et al. (35) also demonstrated that increases of cytosolic Ca^{2+} can be dissociated from cell death by BHP in cultured hepatocytes.

Furthermore, it is unlikely that the early GSSG/ K^+ efflux and intra-cellular accumulation of GSSG is a primary cause of cell death. Further studies are necessary to elucidate the mechanism of BHP-induced cell death.

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