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Phenylbutazone Radicals Inactivate Creatine Kinase

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Creatine kinase (CK) was used as a marker molecule to examine the side effect of damage to tissues by phenylbutazone (PB), an effective drug to treat rheumatic and arthritic diseases, with horseradish peroxidase and hydrogen peroxide (HRP-H_2O_2). PB inactivated CK during its interaction with HRP-H_2O_2, and inactivated CK in rat heart homogenate. PB carbon-centered radicals were formed during the interaction of PB with HRP-H_2O_2. The CK efficiently reduced electron spin resonance signals of the PB carbon-centered radicals. The spin trap agent 2-methyl-2-nitrosopropane strongly prevented CK inactivation. These results show that CK was inactivated through interaction with PB carbon-centered radicals. Sulfhydryl groups and tryptophan residues in CK were lost during the interaction of PB with HRP-H_2O_2, suggesting that cysteine and tryptophan residues are oxidized by PB carbon-centered radicals. Other enzymes, including alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, but not lactate dehydrogenase, were also inactivated. Sulfhydryl enzymes seem to be sensitive to attack by PB carbon-centered radicals. Inhibition of SH enzymes may explain some of the deleterious effects induced by PB.

Keywords: Creatine kinase, horseradish peroxidase, phenylbutazone, phenylbutazone radicals, sulfhydryl enzymes

INTRODUCTION

Phenylbutazone (PB), a nonsteroidal anti-inflammatory drug (NSAID), has analgesic, antipyretic and anti-inflammatory actions that inhibit prostaglandin synthesis by preventing cyclooxygenase activity of prostaglandin H (PGH) synthase.[1] The use of this drug is clinically restricted because of its side effects such as damage of the gastric mucosal membrane and hepatotoxicity.[2,3,4,5]

PGH synthase catalyzes two distinct enzymatic reactions[6,7,8,9]: 1) bis-dioxygenation of arachidonic acid catalyzed by cyclooxygenase activity of PGH synthase to form the hydroperoxy endoperoxide PGG_2; 2) reduction of the hydroperoxide groups of PGG_2 by PGH synthase hydroperoxidase. During PGH synthase-catalyzed reduction of PGG_2, many xenobiotics, including PB, are oxidized by PGH synthase hydroperoxidase through a one electron transfer.[10,11,12] Similarly, horseradish peroxidase (HRP) also oxidizes PB to a PB carbon-centered radical.[13] In addition, Evans et al.[14,15] showed that PB greatly promoted oxidative damage induced by myoglobin or hemoglobin with H_2O_2. However, the exact mechanism of tissue damage induced by this drug remains to be clarified.

Neutrophils, which have a large amount of myeloperoxidase, infiltrate inflammatory tissues. Also the stomach and intestine have perox-

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idases at high levels.[16] These findings strongly suggest that peroxidases are involved in tissue damage induced by NSAIDs. During the metabolism of PB induced by HRP and H_2O_2 (HRP-H_2O_2), PB is oxidized to free radicals by a one electron transfer.[13] However, the action of PB radicals on biological components remains to be clarified.

Creatine kinase (CK, EC 2.7.3.2) is a suitable marker to examine damage induced by PB, because it is very sensitive to oxidative stress[17,18] and is distributed abundantly in tissues. CK has an important role in energy metabolism.[19] The inhibition of this enzyme activity may critically damage tissues. In this study, we show that PB carbon-centered radicals are involved in inactivation of CK induced by PB with HRP-H_2O_2.

MATERIALS AND METHODS

Materials

The following materials were used: PB, CK (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rabbit muscle), lactate dehydrogenase (LDH, porcine muscle) and 2-methyl-2-nitrosopropane (MNP) were obtained from Sigma Chemical Co. St. Louis MO. U.S.A.; HRP was obtained from Wako Pure Chemical Industry, Osaka, Japan; alcohol dehydrogenase (yeast) from Oriental Yeast Co. Ltd, Tokyo, Japan. Other chemicals were analytical grade products obtained from commercial suppliers.

Determination of enzyme activities

The CK activity was measured at 30 °C using a CK kit from Wako Pure Chemical Industries. The CK kit consisted of 22 mM creatine phosphate, 0.89 mM ADP, hexokinase (1.1 u/ml), 0.72 mM NADP and glucose-6-phosphate dehydrogenase (1.1 u/ml) in 80 mM Tris buffer at pH 6.8. CK produces ATP from creatine phosphate and ADP. Hexokinase produces glucose-6-phosphate from ATP and glucose. Finally, glucose-6-phosphate dehydrogenase produces NADPH from glucose-6-phosphate and NADP. The activity of CK was measured by the formation of NADPH at 340 nm. The activity of HRP was determined as follows: the reaction mixture contained 0.27 mM H_2O_2, 1.7 mM KI and HRP in 10 mM acetate buffer at pH 5.0. After incubating for 5 min at 37 °C, the absorbance was measured at 375 nm. The alcohol dehydrogenase (ADH) activity was measured by the method of Bonnischen and Brink[20] and the activity of GAPDH was measured by the method of Prinsz et al..[21] The activity of LDH was measured at 37 °C using a Wako Pure Chemical Industries LDH kit. The protein was determined using the bicinchoninic acid method, as reported by Redinbaugh and Turley.[22]

Preparation of homogenate

Heart homogenates were prepared from five male Wistar strain rats, weighing about 200 g. The hearts were minced and homogenized in 10 mM Hepes buffer containing 0.15 M NaCl at pH 7.4. The homogenates (10%) were dialyzed against the same buffer before use.

Electron spin resonance (ESR) measurement

ESR signals of PB carbon-centered radicals were measured using MNP. The ESR setting was: microwave power, 10 mW; modulation frequency, 100 KHz; modulation field, 0.1 G; receiver gain 2000 and time constant 0.3 sec. The relative signal intensity was measured using Mn^{2+} as a standard.

Amino acid change

The number of sulfhydryl (SH) groups was measured using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) as reported by Ellman et al..[23]

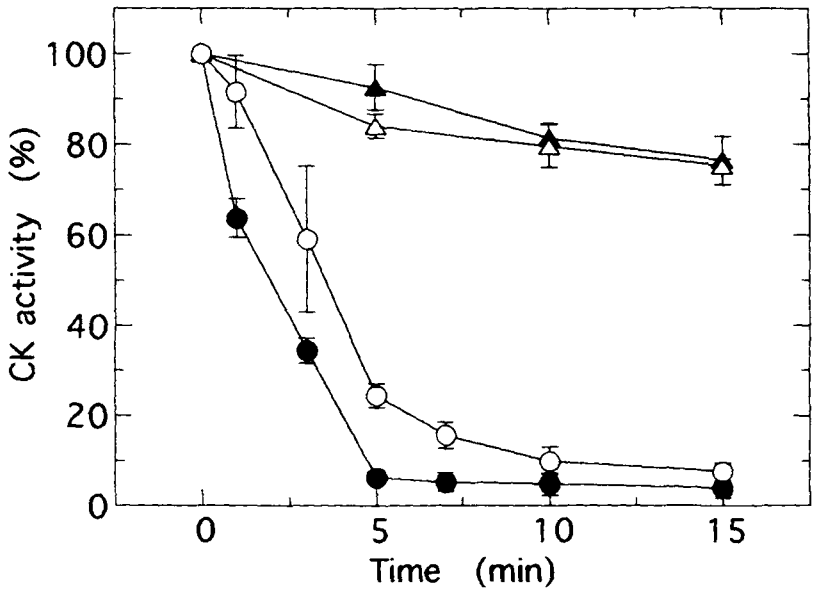

FIGURE 1 Inactivation of CK induced by PB with HRP-H_2O_2. The reaction mixture contained CK (1.23 μ M), 10 μ M H_2O_2 and HRP (0.12 μ M) in 50 mM acetate buffer at pH 5.0. The reaction was started by adding PB. After incubating at 37 °C, the activity of CK was measured as described in the Methods section. For anaerobic experiments, the reaction mixture was purged with nitrogen gas for 10 min, and the reactions were carried out under nitrogen gas. Each point represents the mean ± SD of five experiments. (○), PB added; (▲), without PB; (△), without HRP-H_2O_2 and (•), PB added under anaerobic conditions

The reaction mixture contained 100 μ M PB, 1.2 μ M CK, 0.11 μ M HRP in 50 mM acetate buffer at pH 5.0. Hydrogen peroxide (10 μ M) was added to the reaction mixture. After incubating for 30 min, trichloroacetic acid (0.3%) was added and then the sample was centrifuged for 10 min at 3,000 rpm. The precipitate was solubilized in 1% sodium dodecylsulfate followed by adding 1.0 mM DTNB. After incubating for 30 min, the absorbance was measured at 412 nm. The loss of tryptophan residue was mesured using the fluorescence at 275 nm (ex) and 334 nm (em).[24]

RESULTS

Inactivation of CK

Figure 1 shows that PB rapidly inactivated CK in the presence of HRP with H_2O_2 (HRP–H_2O_2). After incubation for 10 min, the activity of CK was almost lost. CK activity slightly decreased during the incubation in the buffer. In the absence of HRP-H_2O_2, PB caused no CK inactivation. These results show that CK was inactivated by PB activated by HRP-H_2O_2. Under anaerobic conditions, PB inactivated CK more rapidly. Figure 2 shows that inactivation of CK depended on the concentration of PB. The half inhibitory concentration (IC_{50}) was about 2 μ M of the drug. Figure 3 shows that CK was also almost inactivated by PB with HRP-H_2O_2 in heart homogenate. Incubation of the homogenate at pH 5.0 caused rapid inactivation of CK. To avoid this inactivation of CK, we tested the effect of PB on the CK activity in the homogenate at pH 7.4. In the absence of HRP-H_2O_2, PB caused no inactivation of CK. These results show that CK in the homogenate was inactivated during the interaction of PB with HRP-H_2O_2. The IC_{50} of PB with the homogenate was about 5 μ M.

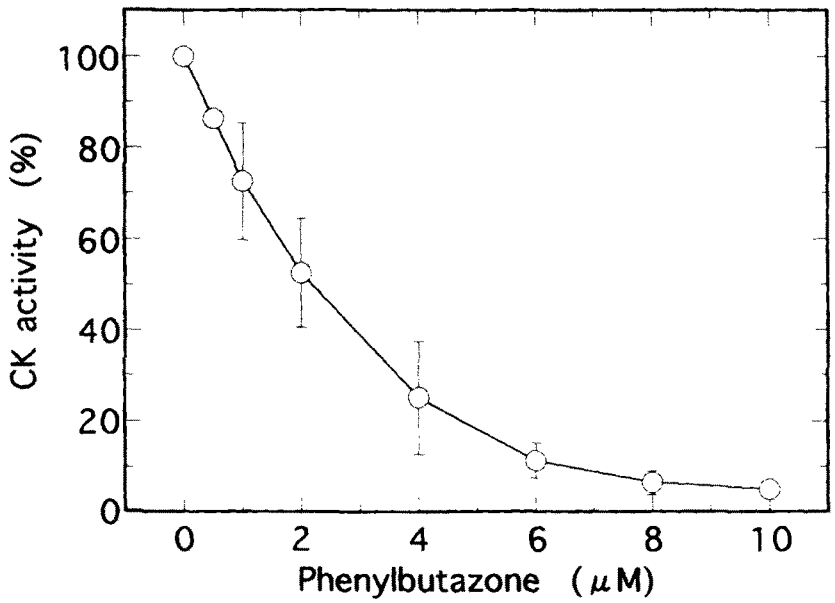

FIGURE 2 Effect of concentrations of PB on CK inactivation. Conditions were the same as described in Figure 1, except for the concentration of PB. After incubating for 10 min, the activity of CK was measured. Each point represents the mean ± SD of five experiments

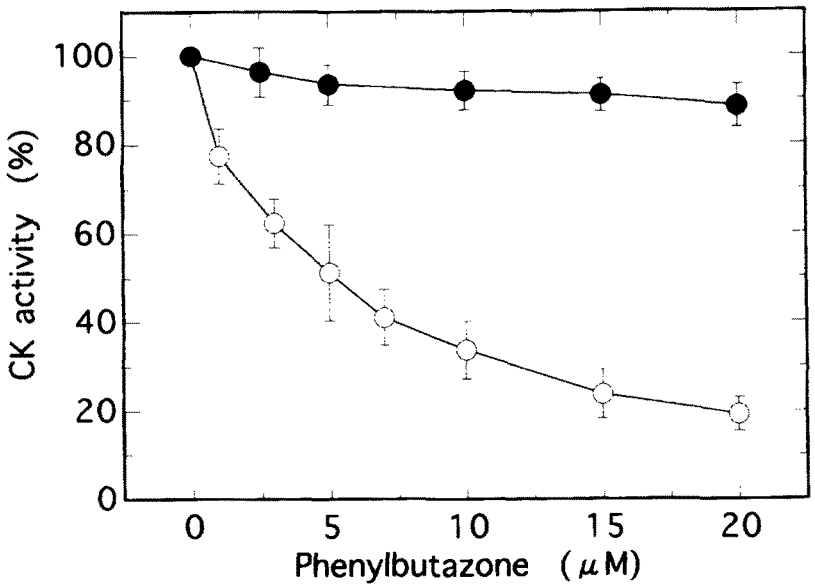

FIGURE 3 Inactivation of CK in heart homogenate induced by PB with HRP-H_2O_2. Heart homogenate (0.1 mg/ml) was suspended in 10 mM Hepes buffer at pH 7.4 containing 0.15 M NaCl and HRP (1.24 μ M) and 50 μM H_2O_2. After incubating with PB for 15 min, the activity of CK was measured. (○), PB added and (•), without PB. Each point represents the mean ± SD of five experiments

TABLE I Loss of SH groups and tryptophan residues in CK during interaction of PB with HRP-H_2O_2

System	*Loss of SH (%)*	*Loss of Trp (%)*
Complete	32.4 ± 1.4	54.7 ± 4.7
-HRP	7.6 ± 3.2	8.7 ± 4.2
-H_2O_2	4.4 ± 2.4	13.5 ± 2.1
-HRP-H_2O_2	2.4 ± 2.2	3.0 ± 3.0

Conditions were the same as described in Figure 1. After incubating for 30 min, loss of SH group and of tryptophan residues was measured as described in the Methods section. Each value represents the mean ± SD of five experiments.

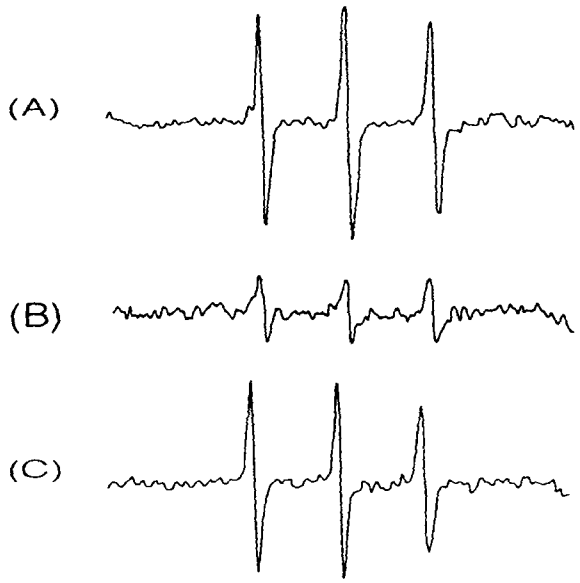

FIGURE 4 ESR spectra of PB carbon-centered radicals formed by PB with HRP-H_2O_2. The complete reaction system consisted of 250 μ M PB, 100 μ M H_2O_2, 2.5 μ M HRP, 10 mM MNP in 10 mM acetate buffer at pH 5.0. Other conditions were as described in the Methods section. A, complete reaction system; B, + CK (12.3 μ M) and C, + bovine serum albumin (15.2 μM). The typical result of three experiments is indicated. The variation was less than 10%

Formation of PB carbon-centered radicals

Figure 4 shows that when PB was incubated with HRP-H_2O_2 in the presence of MNP as a spin trap agent, ESR signals of PB carbon-centered radicals were detected, as reported by Hughes et al..[25] Adding CK strongly diminished the ESR signals of PB carbon-centered radicals. In contrast, bovine serum albumin caused a much lower diminution of the signal. Evidently, CK was very sensitive to the PB carbon-centered radicals. Also, MNP blocked the CK inactivation induced by PB with HRP-H_2O_2 concentration dependently (Figure 5). These results show that CK directly reacts with PB carbon-centered radicals to lose its enzyme activity. Oxygen incorporation into PB was measured, and Figure 6 shows that MNP inhibited the incorporation of molecular oxygen into PB. The oxygen consumption was inhibited by about 25% by MNP at 1.0 mM. These results show that MNP competed with oxygen in PB carbon-centered radicals. The peroxyl radicals may be formed by incorporating oxygen into PB carbon-centered radicals.

Loss of SH groups and amino acid residues

CK is a typical SH enzyme. We therefore examined if CK loses sulfhydryl (SH) groups during the interaction of PB with HRP-H_2O_2. Table I shows that approximately 30% of the SH groups in CK were lost during the interaction of PB with HRP-H_2O_2, whereas without HRP, H_2O_2or HRP-H_2O_2 caused the loss of few SH groups. We tested if tryptophan and tyrosine residues of CK were lost. Of interest, tryptophan residues were very sensitive to PB with HRP-H_2O_2. About 50% of tryptophan residues were diminished by PB with HRP-H_2O_2, whereas without HRP, H_2O_2 or HRP-H_2O_2 only very slightly caused the loss of tryptophan residues. Tyrosine residues were constant (data not shown). These results show that cysteine and tryptophan residues in CK were very sensitive to PB with HRP-H_2O_2.

Inactivation of other SH enzymes by PB

We tested if other enzymes, including ADH, GAPDH and LDH, were also inactivated during the interaction of PB with HRP-H_2O_2. Figure 7 shows that typical SH enzymes of ADH, CK and

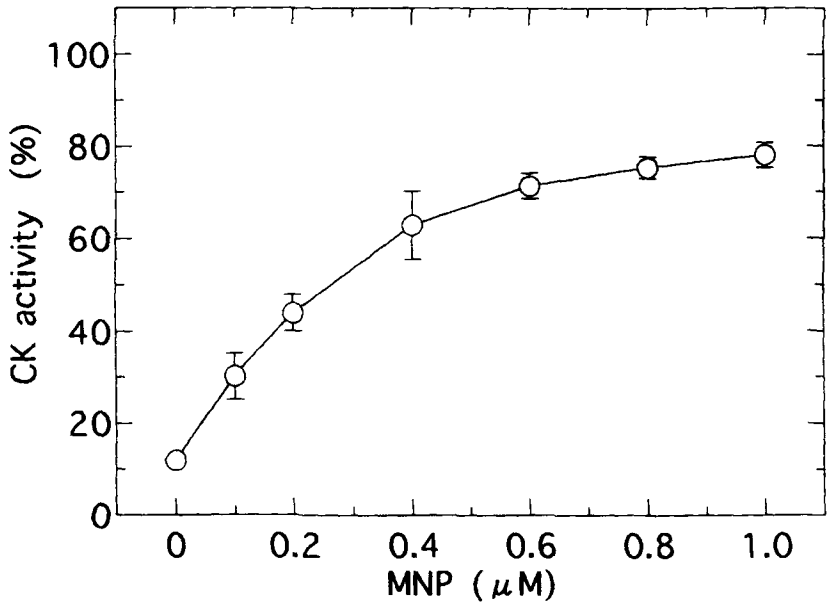

FIGURE 5 Blocking of CK inactivation by MNP. The conditions were the same as described in Figure 1. MNP was added to the reaction mixture. Each points represents the mean ± SD of five experiments

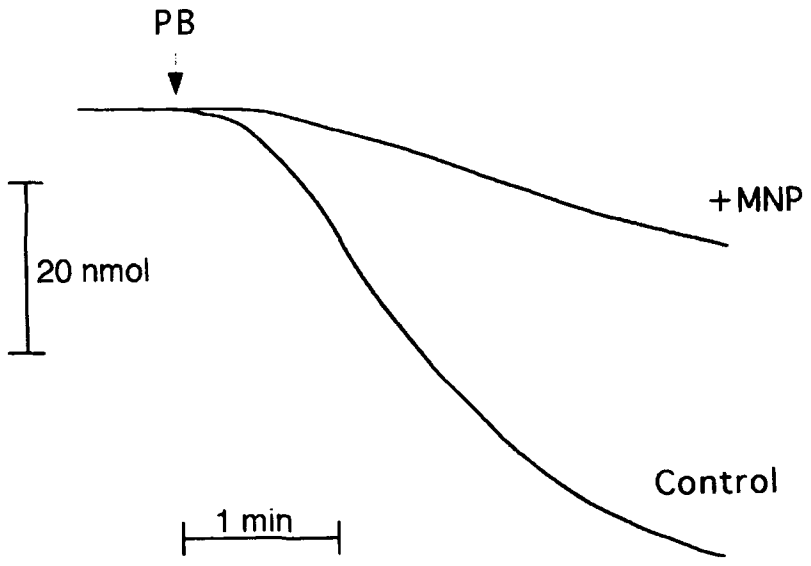

FIGURE 6 Inhibition of oxygen uptake into the PB carbon-centered radical by the spin trap MNP. Conditions were the same as described in Figure 1. The amount of oxygen in solution was measured using an oxygen electrode. The typical results in three experiments was indicated. The variation was less than 10%

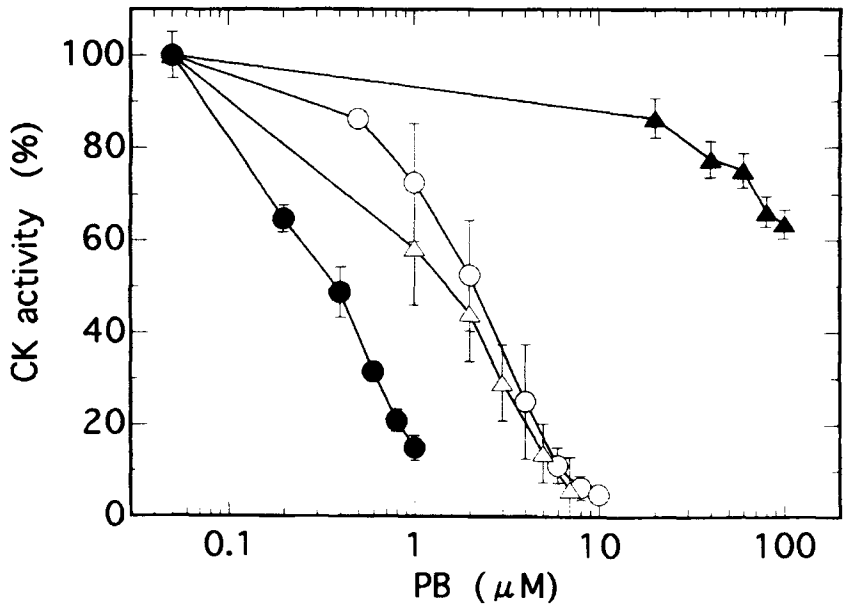

FIGURE 7 Inactivation of various enzymes induced by PB with HRP-H_2O_2. Conditions were the same as described in Figure 1, except for the enzyme. CK (1.23 μ M), ADH (2.9 μ M), GAPDH (2.8 μ M) or LDH (0.7 μ M) were added to the reaction mixture. After incubating for 10 min, the activity of the enzymes was measured. Each points represents the mean ± SD of five experiments. (○), CK; (•), ADH; (△), GAPDH and (▲), LDH

GAPDH were readily inactivated by PB with HRP-H_2O_2. LDH was much less sensitive to PB with HRP-H_2O_2. SH enzymes seem very sensitive to attack by PB carbon-centered radicals and/or the peroxyl radicals.

DISCUSSION

This study showed that PB carbon-centered radicals formed during the interaction with HRP-H_2O_2 caused inactivation of CK. Peroxidases oxidize various compounds by a one electron transfer to form free radical.[26,27,28,29,30] Parij and Neve[26] showed that NSAIDs react with HRP compound II causing its reduction to the native HRP. We confirmed that PB changed the spectrum of a mixture of HRP compounds I and II, which was formed through the interaction of HRP with H_2O_2, to near native HRP (data not shown). These results strongly suggest that PB carbon-centered radicals are formed through oxidation of PB by HRP compounds I or II. Indeed, ESR signals of PB carbon-centered radicals were detected using MNP during the interaction of PB with HRP-H_2O_2. The ESR signals were strongly diminished by adding CK. Evidently, PB carbon-centered radicals directly attacked CK to inactivate CK. However, CK was inactivated under aerobic or anaerobic conditions. Superoxide and hydroxyl radicals seem unlikely to participate in the inactivation of CK because superoxide dismutase, dimethylsulfoxide and mannitol, which are superoxide and hydroxyl radical scavengers, had no effect on the CK inactivation (data not shown). Oxygen was consumed during the interaction of PB with HRP-H_2O_2, suggesting that peroxyl radicals were produced from PB carbon-centered radical under anaerobic conditions. Both PB carbon-cen-

tered radicals and the peroxyl radicals should participate in CK inactivation, because CK was inactivated under aerobic or anaerobic conditions. MNP competed with oxygen to PB carbon-centered radicals, leading to a prevention of CK inactivation. Inactivation of CK was due to a loss of SH groups and tryptophan residues. PB carbon-centered radicals and peroxyl radicals, or both, may attack cysteine and tryptophan residues in CK to inactivate CK.

HRP-catalyzed oxidation of o-dianisidine is inhibited by NSAIDs, including indomethacin, oxyphenylbutazone, phenylbutazone and p-acetaminophenol.[31] Also, NSAIDs, including PB, inhibit hypochlorous acid formation from Cl^- and H_2O_2 by myeloperoxidase[32], suggesting that inhibition of myeloperoxidase partially causes antiinflammatory action. In their study[32], however, the concentration of PB used was higher by a one or two order magnitude than the concentration used in this study. The IC_{50S} of PB that inhibit the activity of pure CK or in the homogenate were about 2 μ M and 5 μ M, respectively. We confirmed that PB had no effect on HRP activity up to 100 μ M. These results suggest that PB was not only antiinflammatory, but was also deleterious with tissues at low levels.

CK is widely distributed in tissues and is generally associated with the physiological role of ATP regeneration in conjunction with the contractile or transport system[19]. The inactivation of CK may seriously retard energy metabolism. ADH and GAPDH, which are typical SH enzymes, were readily inhibited by PB with HRP-H_2O_2. SH enzymes seem to be very sensitive to attack by PB carbon-centered radicals. Inhibition of the SH enzymes may explain some of the deleterious effects induced by PB.

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