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Inactivation of Alcohol Dehydrogenase by Piroxicam-derived Radicals

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Alcohol dehydrogenase (ADH) was used as a marker molecule to clarify the mechanism of gastric mucosal damage as a side effect of using piroxicam. Piroxicam inactivated ADH during interaction of ADH with horseradish peroxidase and H_2O_2 (HRP–H_2O_2). The ADH was more easily inactivated under aerobic than anaerobic conditions, indicating participation by oxygen. Superoxide dismutase, but not hydroxyl radical scavengers, inhibited inactivation of ADH, indicating participation by superoxide. Sulfhydryl (SH) groups in ADH were lost during incubation of piroxicam with HRP–H_2O_2. Adding reduced glutathione (GSH) efficiently blocked ADH inactivation. Other SH enzymes, including creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, were also inactivated by piroxicam with HRP–H_2O_2. Thus SH groups in the enzymes seem vulnerable to piroxicam activated by HRP–H_2O_2. Spectral change in piroxicam was caused by HRP–H_2O_2. ESR signals of glutathionyl radicals occurred during incubation of piroxicam with HRP–H_2O_2 in the presence of GSH. Under anaerobic conditions, glutathionyl radical formation increased. Thus piroxicam free radicals interact with GSH to produce glutathionyl radicals. Piroxicam peroxyl radicals or superoxide, or both, seem to inactivate ADH. Superoxide may be produced through interaction of peroxyl radicals with H_2O_2. Thus superoxide dismutase may inhibit inactivation of ADH through reducing piroxicam peroxyl radicals or blocking interaction of SH groups with O_2^-, or both. Other oxicam derivatives, including isoxicam, tenoxicam and meloxicam, induced ADH inactivation in the presence of HRP–H_2O_2.

Keywords: Piroxicam; Horseradish peroxidase; Alcohol dehydrogenase; Free radical; Sulfhydryl group

INTRODUCTION

Piroxicam, a preferential cyclooxygenase-1 inhibitor, is widely used as an effective nonsteroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic, and antipyretic activities.[1] However, administration of this drug to rats causes severe gastric mucosal damage and multiple intestinal lesions.[1,2] The sequence of events resulting from cyclooxygenase inhibition does not explain the gastric mucosal damage induced by piroxicam.[2,3]

NSAID-induced gastrointestinal injury is reduced by an increase in antioxidant defenses, such as by administering catalase or superoxide dismutase.[4,5] Neutrophil-induced free radicals contribute markedly to the pathogenesis of gastric erosions induced by piroxicam.[6,7] Neutrophils infiltrate inflammatory sites to release myeloperoxidase. The stomach has a large amount of gastric peroxidase.[8] Prostaglandin H synthase (PHS) has not only cyclooxygenase activity, but also peroxidase activity.[9–12] During PHS-catalyzed reduction of prostaglandin G_2, many xenobiotics are oxidized by PHS hydroperoxidase through a one-electron transfer[13–15] to form free radicals. These findings suggest a potential contribution of free radicals, including oxygen radicals, to NSAID-induced gastrointestinal mucosal damage. However, whether free radicals contribute to gastrointestinal damage induced by NSAIDs is not certain.

We previously showed that phenylbutazone[16,17] and indomethacin[18,19] inactivates creatine kinase (CK) and lipid peroxidation of membranes during oxidation by horseradish peroxidase (HRP) in the presence of H_2O_2 (HRP–H_2O_2). In this process, free radicals from phenylbutazone and indomethacin have a crucial role in the damage to biological molecules. However, the mechanisms of membrane

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damage induced by these drugs are very different. Although oxicam-derived compounds are oxidized by peroxidases,[20,21] whether free radicals participate in damage to biological molecules is not clear.

In this study, we examined the inactivation of alcohol dehydrogenase (ADH) induced by piroxicam in the presence of HRP–H_2O_2, and show that free radicals from piroxicam have a crucial role in ADH inactivation.

MATERIALS AND METHODS

Materials

Piroxicam, meloxicam, tenoxicam, isoxicam, CK (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rabbit muscle), lactate dehydrogenase (LDH, porcine muscle) and superoxide dismutase (SOD, bovine blood) were from Sigma Chemical Co. (St. Louis, MO, USA); ADH (yeast) was from Oriental Yeast Co. Ltd. (Tokyo, Japan); HRP, reduced glutathione (GSH) and oxidized glutathione (GSSG) were from Wako Pure Chemical Industry (Osaka, Japan); 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, ultra pure grade) was from Mitsui Toatu Co. Ltd. Other chemicals were analytical grade products from commercial suppliers. Denatured SOD was prepared by autoclaving native enzyme for 30 min.

Measurement of Enzyme Activities

ADH activity was measured by using the method of Bonnichsen and Brink.[22] After ADH was incubated with piroxicam in the presence of HRP–H_2O_2, the enzyme activity was measured by formation of NADH at 340 nm in 0.1 M glycine buffer at pH 9.6 containing 65 mM ethanol and 0.37 mM NAD. The activity of HRP was measured by using the method of Das and Banerjee.[23] The reaction mixture contained 0.27 mM H_2O_2, 1.7 mM KI and HRP in 50 mM acetate buffer at pH 5.0. After the mixture was incubated for 5 min at 37°C, the absorbance was measured at 375 nm. CK and LDH activities were measured by using CK and LDH kits, respectively, from Wako Pure Chemical Industries. The activity of GAPDH was measured by using the method of Prinsz *et al.*[24] Protein was measured by using the bicinchoninic acid method.[25]

Measurement of Sulfhydryl Groups

The number of sulfhydryl (SH) groups was found by using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).[26] The reaction mixture contained piroxicam, 7.1 μM ADH, 0.75 μM HRP, 50.0 μM of H_2O_2 and 0.15 M NaCl in 10 mM Hepes buffer at pH 7.4. After the sample was incubated for 30 min, trichloroacetic acid (10.0%) was added to it and then the sample was centrifuged for 10 min at 3000 rpm. The precipitate was dissolved in 1% sodium dodecylsulfate and then 1.0 mM DTNB was added. After the mixture was incubated for 30 min, the absorbance at 412 nm was measured.

Electron Spin Resonance (ESR) Measurement

ESR signals of glutathionyl radicals were measured in 10 mM Hepes buffer containing piroxicam (1.0 mM), 50 μM H_2O_2, 0.75 μM HRP, 5.0 mM GSH, diethylenetriamine pentaacetic acid (1.0 mM), 0.15 M NaCl and 100 mM DMPO. For anaerobic experiments, the reaction mixture was purged with argon gas for 10 min and the reactions were done under argon gas. The ESR setting was: microwave power, 10 mW; modulation frequency, 100 KHz; modulation field, 0.1 G; receiver gain 1000 and time constant 0.3 s.

RESULTS

Inhibition of ADH

Figure 1 shows the inactivation of ADH induced by piroxicam with HRP–H_2O_2. After piroxicam was incubated for 10 min, loss of ADH activity was 90%. In the absence of HRP–H_2O_2, piroxicam had no effect on ADH activity. A slight loss of enzyme activity occurred during incubation with HRP–H_2O_2 alone. These results indicate that ADH was inactivated by piroxicam activated by HRP–H_2O_2. Activity of ADH more easily decreased under

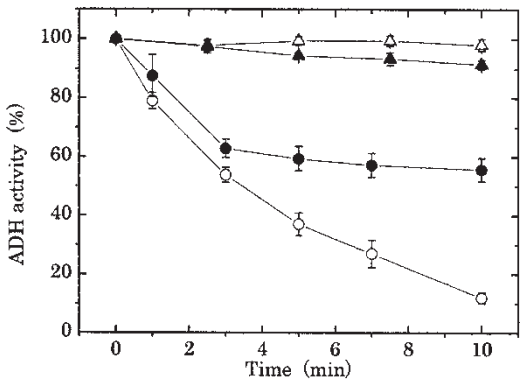

FIGURE 1 Inactivation of ADH induced by piroxicam with HRP–H_2O_2. The complete reaction mixture contained ADH (2.9 μM), 10 μM piroxicam, 0.75 μM HRP, 50 μM H_2O_2 and 0.15 M NaCl in 10 mM Hepes at pH 7.4. The reaction was started by adding piroxicam. After incubation at 37°C, an aliquot of the reaction mixture was removed and the activity of ADH was measured as described in the "Material and Methods" Section. For anaerobic experiments, the reaction mixture was purged with argon gas for 10 min and the reactions were done under argon gas. Each point represents the mean ± SD of five experiments. (○), complete reaction mixture; (▲), without HRP–H_2O_2; (△), without piroxicam and (●), complete reaction mixture under anaerobic conditions.

TABLE I ADH inactivation induced by oxicam derivatives with HRP–H_2O_2

Additions	Concentration (mM)	ADH activity (%)
None		6.0 ± 2.4
+Superoxide dismutase	3.0×10^{-5}	57.7 ± 3.1
+Denatured Superoxide dismutase	3.0×10^{-5}	3.3 ± 1.0
+Catalase	4.5×10^{-6}	58.4 ± 5.8
+Catalase	4.5×10^{-5}	96.7 ± 3.2
+Mannitol	100	6.1 ± 1.7
+Dimethyl sulfoxide	100	5.5 ± 1.8

Conditions were the same as for Fig. 1, except for adding various scavengers. After incubation for 10 min, ADH activity was measured. Each value represents the mean ± SD of five experiments.

aerobic than anaerobic conditions, indicating that oxygen participated in loss of the enzyme activity.

Table I summarizes the effects of oxygen radical scavengers on ADH inactivations. Mannitol and dimethylsulfoxide, which are typical scavengers of hydroxyl radicals, had no effect on the inactivation of ADH, indicating no participation of hydroxyl radicals in the inactivation of ADH. In contrast, catalase completely blocked loss of ADH activity. HRP reacts with H_2O_2 to form complex I or II. Catalase strongly inhibited the inactivation of ADH because the formation of complex I or II was blocked by removal of H_2O_2 (data not shown). Of interest, superoxide dismutase (SOD) inhibited ADH inactivation by about 60%, indicating participation of the superoxide (O_2^-).

Loss of SH Groups

ADH is a typical SH enzyme. We therefore examined if loss of SH groups in ADH was caused by piroxicam with HRP–H_2O_2. Figure 2 shows that SH groups in ADH decreased during oxidation of piroxicam by HRP–H_2O_2. No loss of SH groups was observed during incubation with piroxicam alone. These results indicate that piroxicam activated by HRP–H_2O_2 attacked SH groups to cause loss of enzyme activity. Figure 3 shows the inhibitory effect of GSH on ADH inactivation induced by piroxicam with HRP–H_2O_2. Adding GSH, but not GSSG, efficiently blocked inactivation of ADH dependent upon the concentration. By adding 100 μM of GSH, inactivation of ADH was prevented by about 80 percent.

We examined if other enzymes were inactivated by piroxicam with HRP–H_2O_2. Figure 4 shows that GAPDH and CK, but not LDH, were steadily inactivated by piroxicam with H_2O_2. The IC_{50} of GAPDH and CK was about 6 and 50 μM, respectively. GAPDH and CK are also typical SH enzymes. These results suggest that SH enzymes were vulnerable to piroxicam activated by HRP–H_2O_2.

Participation of Piroxicam Free Radicals

Many drugs[27–31] are one-electron oxidized by peroxidase to form free radicals. Figure 5 shows rapid changes in absorption spectra of piroxicam induced by HRP–H_2O_2. During the oxidation of piroxicam, the absorption peak at 418 nm increased and the peak at 357 nm decreased. The conversion to

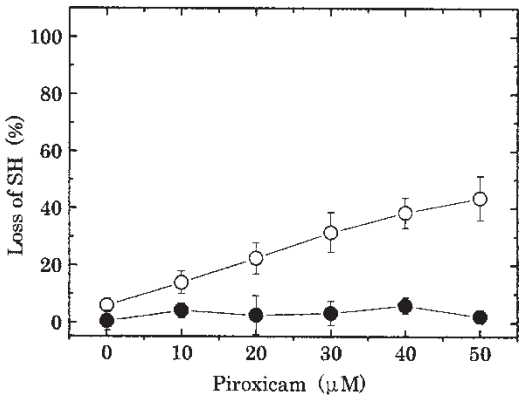

FIGURE 2 Loss of SH groups in ADH induced by piroxicam with HRP–H_2O_2. Conditions were the same as for Fig. 1, except for adding 29 μM ADH. After incubation for 30 min, SH groups were measured by DTNB described in the "Materials and Methods" Section. Each point represents the mean ± SD of five experiments. (○), complete and (●) without HRP–H_2O_2.

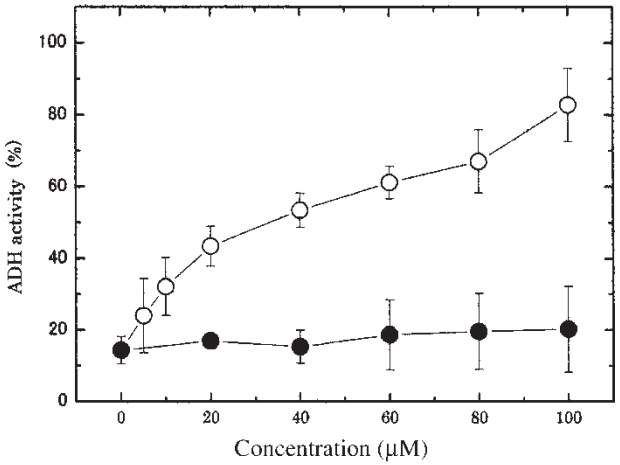

FIGURE 3 Inhibitory effect of GSH on ADH inactivation induced by piroxicam with HRP–H_2O_2. Conditions were the same as for Fig. 1, except for adding GSH. After incubation for 10 min, ADH activity was measured. Each point represents the mean ± SD of five experiments. (○), +GSH and (●), +GSSG.

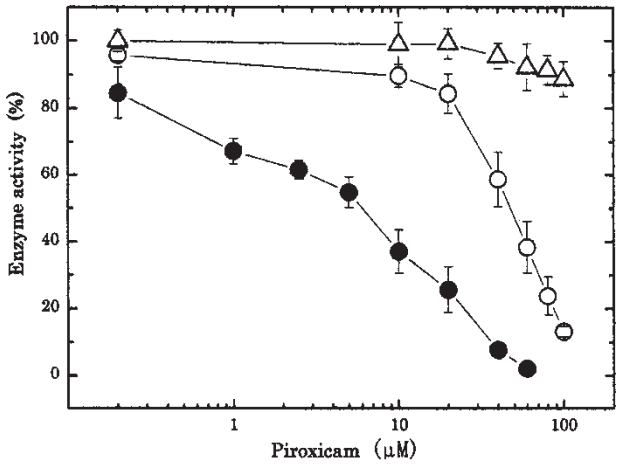

FIGURE 4 Inactivation of various enzymes induced by piroxicam with HRP–H_2O_2. Conditions were the same as for Fig. 1, except for the enzymes. CK (1.23 μM), GAPDH (2.8 μM) or LDH (0.71 μM) was added to the reaction mixture. After incubation for 10 min, the activity was measured. Each point represents the mean ± SD of five experiments. (○), CK; (●), GAPDH and (△), LDH.

its oxidation product generated two isosbestic points at 246 and 405 nm. Omission of HRP or H_2O_2 did not cause a change in spectra of piroxicam. SOD had no effect on the change in absorption of piroxicam at 357 nm, but catalase and GSH strongly inhibited the change in absorption of piroxicam (data not shown).

We tried to detect ESR signals of piroxicam free radicals during the interaction of piroxicam with HRP–H_2O_2. However, we failed to detect piroxicam free radicals even when DMPO or PBN (*N-tert*-butyl-α-phenylnitrone) was used as a spin trap agent. These agents did not affect the change in spectra of piroxicam induced by HRP–H_2O_2 (data not shown). These results suggest that the piroxicam radical is too unstable to be detected by ESR or that spin trap agents cannot react with piroxicam radicals, or both. During incubation with piroxicam with HRP–H_2O_2, the SH groups in ADH were lost and GSH efficiently blocked inactivation of ADH, strongly suggesting that GSH acts as an efficient scavenger of piroxicam free radicals to form glutathionyl radicals. Figure 6 shows formation of glutathionyl radicals. When piroxicam was incubated with HRP–H_2O_2 in the presence of GSH and DMPO, ESR signals ($a_N = 15.0$ G, $a_H = 16.3$ G) consistent with a DMPO-glutathionyl radical adduct were detected. No signals were detected in the absence of GSH and HRP. Weak ESR signals were detected when piroxicam or H_2O_2 was removed from the reaction mixture. The weak ESR signals were detected even when heat-denatured HRP was replaced by native HRP. Possibly, GSH was slightly autoxidized to glutathionyl radicals by hemin. However, the formation of glutathionyl radicals extremely accelerated during the interaction of piroxicam with native HRP and H_2O_2. These results suggest that formation of glutathionyl radicals efficiently increased through interaction of GSH with piroxicam radicals. Adding SOD had little effect on the formation of

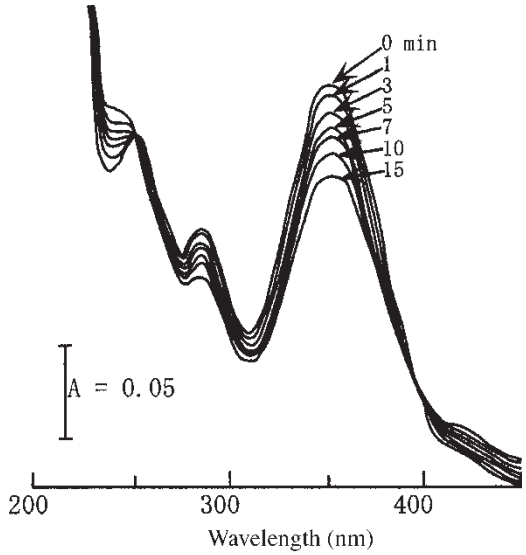

FIGURE 5 Changes in absorption spectra of piroxicam induced by HRP–H_2O_2. Piroxicam was oxidized by HRP (0.75 μM) and H_2O_2 (100 μM) in 10 mM Hepes buffer containing 0.15 M NaCl at pH 7.4. Spectra were recorded at 37°C. The number of the curves is the incubation time (min).

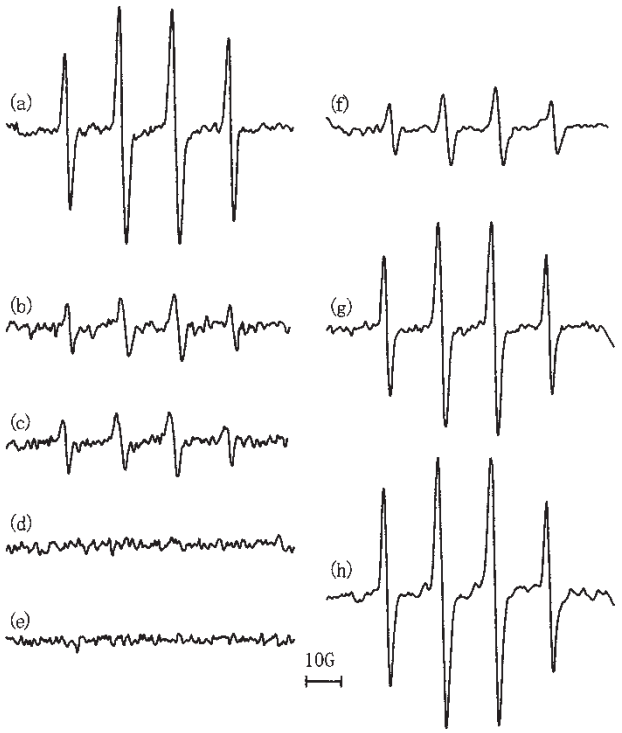

FIGURE 6 Formation of glutathionyl radicals by piroxicam with HRP–H_2O_2 in the presence of GSH. ESR signals were recorded under the conditions described in "Materials and Methods" Section: (a) complete reaction mixture; (b) without piroxicam; (c) without H_2O_2; (d) without GSH; (e) without HRP; (f) a replaced by heat-denatured HRP; (g) complete reaction mixture with SOD (3×10^{-4} mM) and (h) complete reaction mixture under anaerobic conditions.

TABLE II Effect of oxygen radical scavengers on ADH inactivation

Additions	ADH activity (%)	Inhibition (%)
None	91.5 ± 1.5	8.5
Piroxicam	11.9 ± 8.8	88.1
Isoxicam	38.6 ± 13.8	61.4
Tenoxicam	3.5 ± 4.5	96.5
Meloxicam	37.2 ± 10.4	62.8

Conditions were the same as for Fig. 1 except for the oxicam derivatives. Each value represents the mean ± SD of five experiments.

ESR signals of the glutathionyl radical, but the formation of ESR signals increased under anaerobic conditions. Presumably, GSH competes with oxygen against piroxicam radicals under aerobic conditions.

We tested in Table II if other oxicam compounds, including isoxicam, tenoxicam and meloxicam, also inactivated ADH in the presence of HRP–H_2O_2. Figure 7 shows structures of oxicam compounds used in this study. Among oxicam compounds used, tenoxicam was the most efficient drug to inhibit ADH. The order of ability to inhibit ADH was: tenoxicam > piroxicam > meloxicam > isoxicam.

DISCUSSION

This study showed that piroxicam rapidly inactivates ADH during interaction with HRP–H_2O_2. Inactivation of ADH was depressed under anaerobic conditions. Evidently, oxygen increased the deleterious effect of piroxicam on ADH. Our data indicate that O_2^-, but not hydroxyl radicals, participated in ADH inactivation induced by piroxicam with HRP–H_2O_2 because SOD, but not hydroxyl radical scavengers, inhibited ADH inactivation.

GSH is an important constituent of intracellular protective mechanisms against a number of noxious stimuli, including oxidative stress. Both nonprotein and protein SH groups decrease in gastric tissue during administration of NSAIDs.[32–34] In this study, SH groups in ADH were particularly vulnerable to piroxicam activated by HRP–H_2O_2 and GSH efficiently blocked inactivation of ADH. These results suggest that these SH groups are a target of piroxicam activated by HRP–H_2O_2. Phenylbutazone and indomethacin radicals efficiently inactivate SH enzymes.[18,19] GSH is an efficient scavenger of phenylbutazone radicals.[16]

Isoxicam is a poor substrate for hepatic cytochrome P-450 enzymes, but it is easily metabolized by HRP and H_2O_2.[20] They suggested that 3-hydroperoxyl intermediate isoxicam is formed during oxidation of HRP–H_2O_2. Isoxicam also inactivates ADH in the presence of HRP–H_2O_2. We speculate here that the C-3 peroxyl radical of piroxicam, rather than piroxicam free radicals, participated in the inactivation of ADH (Fig. 8). ADH or other enzymes may be inactivated by oxidation of the SH groups by these free radicals. GSH blocked ADH inactivation and changes in the spectrum of piroxicam, indicating that it scavenges piroxicam radicals to produce glutathionyl radicals.

Our data strongly suggest that O_2^- was produced during interaction of piroxicam with HRP–H_2O_2 because SOD inhibited the inactivation of ADH. SOD blocks gastric lipid peroxidation induced by indomethacin.[35,36] Indomethacin radicals react with H_2O_2 to form O_2^-.[17] Superoxide by itself does not initiate lipid peroxidation. In lipid peroxidation induced by indomethacin with HRP–H_2O_2, the O_2^- participated in the lipid peroxidation reaction through reduction of ferric ion. In a reaction system that included indomethacin and HRP–H_2O_2, the O_2^- was produced through interaction of indomethacin radicals with H_2O_2. In this study, O_2^- should have

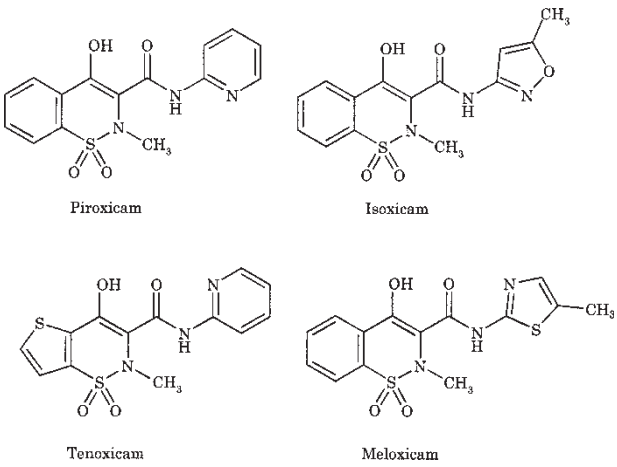

FIGURE 7 Structures of oxicam compounds.

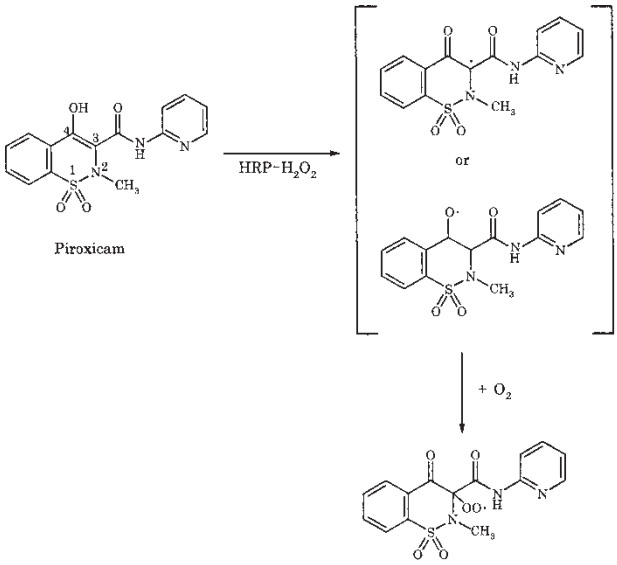

FIGURE 8 Scheme showing oxidation of piroxicam.

been formed through a similar mechanism by which piroxicam peroxyl radicals (POO·) interact with H_2O_2:

$$POO\cdot + H_2O_2 \rightarrow POO^- + 2H^+ + O_2^-$$

SOD converts O_2^- to H_2O_2 and O_2. Presumably, SOD inhibits the inactivation of ADH because of the reduction in formation of peroxyl radicals or the blocking of the interaction of SH groups by O_2^-, or both.

Meloxicam, a new NSAID of the oxicam family, has much greater affinity for cyclooxygenase-2 than for cyclooxygenase-1. This limited selectivity has led to meloxicam being described as a preferential cyclooxygenase-2 inhibitor. However, meloxicam induces gastric lesions associated with an increase in oxidative metabolism.[37] In this study, meloxicam inactivated ADH. Free radicals formed from meloxicam with HRP–H_2O_2 seem to participate in mucosal damage.

Acknowledgements

This study suggests that free radicals formed by peroxidase participate in mucosal damage induced by piroxicam. Oxidative damage of proteins may lead to faulty cell functions.

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