Effects of 1-[(2-Thiazolin-2-yl)amino]acetyl-4-(1,3-dithiol-2-ylidene)-2,3,4,5-tetrahydro-1H-1-benzazepin-3,5-dione Hydrochloride (KF-14363) on Active Oxygen Production

Ikufumi Yoshitake and Kazuhiro Kubo

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari, Nagaizumi-cho, Santo-gun, Shizuoka 411, Japan

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ABSTRACT—The effects of KF-14363 on active oxygen production and membrane stabilization were studied. KF-14363 did not affect hypotonic hemolysis (10% and 70%) and did not inhibit lipid peroxide production induced by t-butyl hydroperoxide at concentrations of less than 100 μM. KF-14363 significantly inhibited active oxygen production in peritoneal exudate cells (PEEC) stimulated with arachidonic acid, A23187 and carbon tetrachloride (CCl₄) at concentrations over 10 μM, 100 μM and 1 μM, respectively. It tended to inhibit formyl-methionyl-leucyl-phenylalanine-stimulated production of active oxygen in PEEC at concentrations over 10 μM, but there was no significant difference owing to large dispersion. Superoxide dismutase (SOD, 10⁴ U/ml) significantly inhibited CCl₄-stimulated production of active oxygen in PEEC. KF-14363 inhibited the radical production from CCl₄ in the presence of a 9000 × g supernatant fraction of the rat liver which was administered with enzyme induction compounds (S9 mix). SOD (10⁴ U/ml) was not effective in this system. In conclusion, KF-14363 inhibited active oxygen production in PEEC induced by various stimulants and also the radical formation from CCl₄ in the presence of S9 mix solution.

Various factors have been reported to induce hepatic injury (1–3), and active oxygen is thought to have the most important role among them (4–6). Physiologically, oxygen produces O₂ (superoxide), H₂O₂, OH (hydroxyl radical) or other active oxygen species. Active oxygen is reactive, producing membrane lipid peroxidation and damages membrane functions (7). In this study, the effects of 1-[(2-thiazolin-2-yl)amino]acetyl-4-(1,3-dithiol-2-ylidene)-2,3,4,5-tetrahydro-1H-1-benzazepin-3,5-dione hydrochloride (KF-14363, Fig. 1), which inhibited hepatic damages in various experimental models, on active oxygen and membrane stabilization was investigated to clarify its mechanism of action.

MATERIALS AND METHODS

Drugs and reagents

KF-14363 (Lots BH-2686, BH-2920-1 and BH-3066) and malotilate (Lots BH-2626 and BH-3082) were synthesized at Kyowa Hakko. Gomicin A (Lot BH-2242) was extracted from the ripe fruits of Schizandra Chinensis Baill at Kyowa Hakko. These drugs were dissolved in dimethylsulfoxide (DMSO) or distilled water to concentrations of 1 × 10⁻⁶ M to 1 × 10⁻⁴ M. A control group was given the vehicle in the same volume as the test drug.
The reagents used in this study were as follows: DMSO (special class, Kanto Chemical Co., Inc.), sodium chloride (special class, Wako Pure Chemical Industries), sodium azide (special class, Nakai Chemical Co., Inc.), t-butyl hydroperoxide (t-BHP, Sigma Co.), trichloroacetic acid (TCA, special class, Kanto Chemical Co., Inc.), 2-thiobarbituric acid (Nakai Chemical Co., Inc.), sodium arsinite (Kanto Chemical Co., Inc.), Dulbecco’s phosphate buffer salts (Flow Co.), casein from milk (special class, Kanto Chemical Co., Inc.), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol, Sigma Co.), arachidonic acid (AA, Sigma Co.), A23187 (Hoechst, Lot 405075), formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma Co.), carbon tetrachloride (CCl4, Kanto Chemical Co., Inc.), superoxide dismutase (SOD, Sigma Co.) and S9 mix solution (Oriental Yeast Co., Lot 90020104).

**Experimental methods**

**Stabilizing effect on rat erythrocyte membranes (8):** Blood samples were taken from male Sprague-Dawley rats (Clea Japan, Inc.) weighing 250 to 300 g, mixed with heparin and centrifuged at 500 X g (Hitachi 05PR-22 model) for 15 min to obtain the erythrocytes. The erythrocyte hemolysis test was conducted according to the Parpart method (9). NaCl concentration was adjusted to induce 10% and 70% hemolysis, and phosphate buffer containing the appropriate amount of NaCl was prepared. Test drugs of various concentrations were dissolved in phosphate buffer to a range of concentrations from 1 X 10⁻⁶ M to 1 X 10⁻⁴ M. Phosphate buffer containing 1% DMSO (the vehicle for water-insoluble drugs) was also prepared. For the control group, phosphate buffer containing 1% DMSO or water was used for the vehicle. A 5-ml aliquot of the test drug or control buffer solution (2 samples for each of the treated groups and 2 samples for the control group) containing 25 μl rat erythrocytes was incubated at 37°C for 1 hr and then centrifuged at 900 X g (Hitachi 05PR-22 model) for 5 min. The 540-nm absorbance of the supernatant was determined using a spectrophotometer (Shimadzu, UV-180).

**Inhibitory effect on lipid peroxide production in rat erythrocytes (10-12):** Erythrocyte samples prepared as described above were diluted to a 5% suspension in 0.15 M phosphate buffer containing 5 mM sodium azide. Various test drug solutions or distilled water was added to 1 ml of the 5% erythrocyte suspensions. Thirty minutes later, the same volume of 1 mM t-BHP was added and the mixture heated for 1 hr. Twenty eight percent TCA containing 0.1 M sodium arsenite was added to stop the reaction. This solution was centrifuged at 1400 X g (Hitachi 05PR-22 model) for 10 min. A 2-ml aliquot of the supernatant was reacted with 0.5 ml of 1% 2-thiobarbituric acid and the color-developed for 15 min in a boiling bath. The absorbance at 532 nm was determined using a spectrophotometer (Shimadzu UV-180).

**Inhibitory effect on active oxygen production in rat peritoneal exudate cells (PEEC):** Phosphate-buffered saline (PBS) containing 1% casein was intraperitoneally administered to male Sprague-Dawley rats (Clea Japan, Inc.) weighing 250 to 300 g, at 120 ml/kg body weight. Sixteen hours later, the rats were sacrificed by bleeding. After the intraperitoneal administration of 20 ml of ice-cold PBS, the rats were laparotomized, the PBS collected and centrifuged at 210 X g (Hitachi 05PR-22
model) for 10 min at 4°C. Cells were suspended in PBS containing Ca\(^{2+}\), Mg\(^{2+}\) and glucose to a density of 5 \times 10^6 cells/ml. Chemiluminescence (CL) was determined according to the method of Mibu et al. (13). A470-\(\mu\)l aliquot of cell suspension was added to 10 \(\mu\)l luminol solution and heated at 37°C for 10 min. Ten microliters of stimulant, AA (200 \(\mu\)M), A23187 (10 \(\mu\)M), fMLP (10 \(\mu\)M) or CCl\(_4\) (0.2 M), was added and CL was determined at 37°C as a function of time using a photon counter (Berthold multi-biolumat, LB9509C). The height of the CL peak was considered to be an indicator of drug-induced inhibition. Cell viability was evaluated by trypan blue staining.

### Inhibitory effect on radical formation from CCl\(_4\) in the presence of S9 mix solution:

The S9 (a rat liver 9000 \(\times\) g supernatant fraction) mix solution (0.47 ml) was mixed with 10 \(\mu\)l of luminol solution and 10 \(\mu\)l of the test drug and heated at 37°C for 10 min. CCl\(_4\) was then added, and CL was determined using a photon counter. The height of the CL peak was considered to be an indicator of drug-induced inhibition. The components in 10 ml of the S9 mix solution were 8 \(\mu\)mol MgCl\(_2\), 33 \(\mu\)mol KCl, 5 \(\mu\)mol glucose-6-phosphate, 4 \(\mu\)mol NADPH, 4 \(\mu\)mol NADH, 100 \(\mu\)mol Na\(_2\)HPO\(_4\) and 100 \(\mu\)mol NaH\(_2\)PO\(_4\).

### Statistical analysis

Values are expressed as the mean or mean ± standard error. In statistical analysis, \(P < 0.05\) by Dunnett's multiple comparison test was considered significant.

### RESULTS

#### Stabilizing effect on rat erythrocyte membrane

Stabilizing effects of three drugs on erythrocyte membranes were examined. KF-14363 did not inhibit 10% or 70% hemolysis at concentrations of less than 100 \(\mu\)M. Gomicin A at 100 \(\mu\)M inhibited 70% hemolysis by 14% compared with the controls and stabilized erythrocyte membranes. Malotilate dose-dependently stabilized erythrocyte membranes and inhibited 10% and 70% hemolysis by approximately 75% and 58% compared with the controls, respectively.

### Inhibitory effect on lipid peroxide production in rat erythrocytes

The inhibitory effect of KF-14363 on t-BHP-induced lipid peroxidation in rat erythrocytes was examined. The concentration of thiobarbituric acid reactive substances in the control group was 4.41 nmoles/mol and 4.73, 4.56 and 4.57 nmoles/ml in the KF-14363 groups at 1, 10 and 100 \(\mu\)M, respectively. KF-14363 did not inhibit t-BHP-induced lipid peroxidation at concentrations below 100 \(\mu\)M.

### Inhibition of active oxygen in rat PEEC

Rat neutrophils were incubated with KF-14363 at 37°C for 20 min to investigate their viability. The viability of rat neutrophils treated by KF-14363 at 100 \(\mu\)M was about 95%. Twenty minutes are equivalent to the time required to activate oxygen production from neutrophils in the presence of various stimulants.

Figure 2 shows representative time-course changes in CL from AA-active PEEC. CL peaked at about 1 min after AA addition. Activated oxygen production decreased thereafter and the CL value almost recovered to the pre-activation values by 5 min after AA addition. The heights of the CL peak in 4 PEEC samples are summarized in Fig. 3. CL in the control group was 4.96 \(\times\) 10⁷ cpm. PEEC treated with KF-14363 at 10 and 100 \(\mu\)M significantly decreased active oxygen to 3.09 \(\times\) 10⁷ and 2.60 \(\times\) 10⁷ cpm, respectively.

Representative time-course CL changes in A23187-stimulated PEEC are shown in Fig. 4. Figure 5 summarizes the effect of KF-14363 on CL production in 4 samples of A23187-stimulated PEEC. KF-14363 slightly decreased CL production at 1 and 10 \(\mu\)M to 18.5 \(\times\) 10⁶ and 12.3 \(\times\) 10⁶ cpm, respectively, compared with that in the control group (24.0 \(\times\) 10⁶ cpm) and significantly decreased it to 8.5 \(\times\) 10⁶ cpm at 100 \(\mu\)M. Stimulation with fMLP induced 2 CL peaks in PEEC (Fig. 6). CL in the vehicle-
added control group was $9.74 \times 10^6$ cpm in the former peak and $9.25 \times 10^6$ cpm in the latter. KF-14363 addition at 10 and 100 $\mu$M decreased the former peak to $6.72 \times 10^6$ and $6.52 \times 10^6$ cpm and the latter to $6.58 \times 10^6$ and $5.69 \times 10^6$ cpm, respectively, but the difference was not significant, with large dispersion. Figure 7 shows the pattern of CCL-induced production of activated oxygen in PEEC. KF-14363 at concentrations over 1 $\mu$M significantly inhibited the production of active oxygen ($8.98 \times 10^7$ cpm, $P < 0.05$) (Fig. 8).
Fig. 6. Actual record of the inhibition by KF-14363 of formyl-methionyl-leucyl-phenylalanine-induced luminol dependent chemiluminescence from peritoneal exudate cells of rats. Peritoneal exudate cells (2.4 X 10⁶) were first incubated with luminol (56.4 nmol) and KF-14363 (1–100 μM) for 10 min at 37°C and then formyl-methionyl-leucyl-phenylalanine (10⁻⁵ M) was added.

Fig. 7. Actual record of the inhibition by KF-14363 of carbon tetrachloride-induced luminol dependent chemiluminescence from peritoneal exudate cells of rats. Peritoneal exudate cells (2.4 X 10⁶) were first incubated with luminol (56.4 nmol) and KF-14363 (1–100 μM) for 10 min at 37°C and then carbon tetrachloride (0.2 M) was added.

Fig. 8. Effects of KF-14363 on carbon tetrachloride-induced luminol dependent chemiluminescence from peritoneal exudate cells of rats. Each value shows the mean ± S.E. of 4 independent experiments. *, significantly different from the control, P < 0.05; **, significantly different from the control, P < 0.01.

Fig. 9. Actual record of the inhibition by KF-14363 of luminol dependent chemiluminescence induced by carbon tetrachloride in the presence of S9 mix solution. S9 mix solution (0.47 ml) was first incubated with luminol (56.4 nmol) and KF-14363 (1–100 μM) for 10 min at 37°C and then carbon tetrachloride (0.2 M) was added.
The inhibitory effect was more potent at higher doses. The addition of $10^4$ U/ml SOD, which scavenges superoxide, significantly decreased CL to $2.54 \times 10^7$ cpm.

**Inhibitory effect on radical formation from CCl₄ in the presence of S9 mix solution**

CCl₄ addition to S9 mix solution containing cofactor produced radicals as shown in Fig. 9 and this peaked after 1 min. KF-14363 significantly decreased CL at concentrations above 10 μM (P < 0.05). SOD at $10^4$ U/ml did not decrease CL (Fig. 10).

**DISCUSSION**

Membrane stabilizing and active oxygen scavenging effects have been reported as mechanisms of action for liver protection agents effective in drug-induced models of hepatic injury (14, 15). In this study, the effects of KF-14363, which is effective in various experimental models of hepatic injury, on active oxygen and membrane stabilization were examined in order to clarify the mechanisms of liver protection. Membrane stabilizing effects were studied in hypotonic erythrocyte hemolysis. The reference drug malotilate dose-dependently inhibited hemolysis. Kanoh et al. (14) reported that malotilate exhibited maximum inhibition of incubated erythrocyte hemolysis at 10 μM. In our hypotonic erythrocyte hemolysis test, malotilate inhibited hemolysis at concentrations over 10 μM, which almost coincided with their report. Gomicin A, another reference drug, inhibited 70% hemolysis. Suzuki et al. (15) reported that gomicin A inhibited erythrocyte hemolysis at concentrations between $10^{-8}$ M and $10^{-5}$ M. The effect in our study seemed to be slightly less than that in their report.

The effects of KF-14363 on active oxygen scavenging and the inhibition of lipid peroxidation in erythrocytes were studied using t-BHP, an organic peroxide producing alkoxyl (t-BuO·) and peroxyl (t-BuOO·) radicals as reactive as the hydroxyl radical (·OH) (16), but this effect was not observed. This finding indicates that KF-14363 does not scavenge these radicals.

However, as the possibility remained that KF-14363 inhibited active oxygen production in erythrocytes, active oxygen production of PEEC in rats was studied using 4 stimulants, AA (17), A23187 (18), fMLP (19-21) and CCl₄. Calcium is purported to be an important factor in neutrophil activation (22, 23). Increased intracellular Ca levels are thought to activate phospholipid metabolism, release AA metabolites and produce active oxygen during metabolism by the cyclooxygenase and lipoxygenase routes (24). Yoshimoto et al. (25) re-
ported that the cyclooxygenase inhibitor indomethacin did not inhibit CL, which suggests that active oxygen is produced by the lipooxygenase route. As KF-14363 inhibited AA- and A23187-induced CL, it may inhibit the production of active oxygen by this route. Since the viability of PEEC treated by KF-14363 (100 μM) was about 95%, these inhibitory effects on the production of active oxygen are not caused by the death of PEEC. CCl_{4} has been reported to increase intracellular Ca^{2+} accumulation in hepatic cells (26, 27). Therefore, CCl_{4} may also produce active oxygen by increasing intracellular Ca^{2+}, activating phospholipid metabolism and releasing AA metabolites. Since the CCl_{4}-induced production of active oxygen in PEEC was inhibited by SOD, it seems to be a superoxide. Chemotactic substances like fMLP have been also reported to produce active oxygen (18). The stimulation of neutrophils with fMLP produces LTβ_{4} (28), which produces a superoxide (29, 30). Therefore, fMLP is also thought to activate the AA cascade and produce active oxygen. The effect of KF-14363 was widely dispersed and thought not significant, and inhibitory tendency was recognized. Consequently, it is suggested that KF-14363 acts on the AA cascade and inhibits active oxygen production. The P. acnes + LPS model of hepatic injury in mice is induced by hepatic injury factors released from adherent cells accumulated in the liver (31–33), and KF-14363 was effective in this model. The effect of KF-14363 on PEEC in this study suggests that it inhibits the production of injurious factors (active oxygen, etc.) in hepatic adherent cells and thus protects the liver. KF-14363 was also effective in CCl_{4}-induced hepatic injury models in vivo in mice and rats. Inhibitory effects on active oxygen production in PEEC are likely to be one of mechanisms of action for the liver protecting effect of KF-14363 in vivo.

As for the mechanism of CCl_{4}-induced hepatic injury, the argument that CCl_{4} is metabolized by drug metabolizing enzymes in hepatic microsomes and converted into °CCl_{3}, which triggers peroxidation of unsaturated fatty acids, thus inducing cell membrane injury (34, 35), is regarded as important. Since metabolism of CCl_{4} by hepatic microsomes and the determination of radicals by CL has already been published (36), rat hepatic S9 mix solution was added with cofactor, and CCl_{4} was metabolized in this study. As a result, a radical was determined as CL. Because SOD failed to inhibit the CL, it is not a superoxide. The CCl_{4}-induced CL production in PEEC could be inhibited by SOD, but in S9 mix, it could not. This difference may exist because there is cell membrane in PEEC, but the preparation is cell membrane free in S9. Namely, the active oxygen (superoxide) on the cell membrane of PEEC that results from a series of reactions is produced by CCl_{4}. On the other hand, S9 mix (includes microsomes) metabolizes CCl_{4} into °CCl_{3} and CL may be produced by °CCl_{3}, °CCl_{3}O_{2} or perox radicals, but the possibility that the CL may be produced by another radical cannot be denied. As KF-14363 also inhibited radical production, its inhibitory effect on CCl_{4}-induced hepatic injury (in vivo) is very likely attributable to the inhibition of °CCl_{3} production by hepatic microsomes. However, the mechanism of inhibiting active oxygen production in PEEC may also be partly involved in the inhibition of CCl_{4}-induced hepatic injury in vivo.

The findings described above indicate that KF-14363 does not stabilize membranes nor does it scavenge active oxygen produced by t-BHP stimulation. KF-14363 was proven to inhibit active oxygen production in PEEC induced by various stimulants as well as radical formation from CCl_{4} in the presence of S9 mix solution. These effects contribute to liver protection by KF-14363 in various experimental models of hepatic injury.

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