Interaction of BW755C, a potent inhibitor of lipoxygenase and cyclo-oxygenase, with mitochondrial cytochrome oxidase[1]

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Summary. The interaction between BW755C (3-amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline), a potent inhibitor of both lipoxygenase and cyclo-oxygenase, and respiratory chain in mitochondria and electron transport particles (ETP) from rat livers was examined. BW755C accelerated the oxygen uptake by mitochondria without the addition of substrate for the respiratory chain. Spectrophotometric study revealed that BW755C was quickly oxidized by cytochrome oxidase in mitochondria to a compound possessing an absorption maximum at 524 nm. p-Phenylenediamine (p-diaminobenzene, PPDA), which, like BW755C, serves as an electron donor to cytoschrome oxidase, was shown to inhibit the generation of active oxygen in macrophages; the inhibition was stronger than that of BW755C. These results strongly suggest that the oxidative conversion of BW755C by mitochondrial cytochrome oxidase is associated with its potentially inhibitory action on the active oxygen-generating system in phagocytes.

Key words. Rat liver; cytochrome oxidase, mitochondria; BW755C; 3,amino-1-[m-(trifluoromethyl)phenyl]-2-pyrazoline; lipoxygenase inhibitor; cyclo-oxygenase inhibitor.

It is now well accepted that prostaglandins contribute to the inflammatory response and that several nonsteroidal anti-inflammatory drugs (NSAIDs) depress the synthesis of prostaglandins by inhibiting cyclo-oxygenase in the arachidonic cascade, which is considered to be a mechanism for this anti-inflammatory activities[5,6] (and references cited therein). Recently, BW755C has been demonstrated to elicit inhibitory effects on both lipoxygenase and cyclo-oxygenase[7], but the precise molecular mechanism for the inhibition of these enzymes is unknown. Several possible modes of action of BW755C are proposed; action as a radical scavenger, direct interaction with these oxygenases, or inhibition of the active oxygen-generating system. It is of importance to elucidate the precise mode of action of this drug for understanding the mechanism of pharmaceutical actions of NSAIDs.

Most recently, we have found that BW755C is able to donate electrons to cytochrome oxidase in mitochondria. Little is known regarding the correlation between the redox reaction with mitochondrial cytochrome oxidase and the inhibitory effect on prostaglandin synthesis. But evidence is given for such a correlation by the finding that PPDA, an electron donor to cytochrome oxidase[8] like BW755C, also inhibits the generation of active oxygen in macrophages, and also by the finding that cytochrome(s) participate in the active oxygen-generation of phagocytes[9-11]. Therefore it is not unreasonable to expect a redox interaction BW755C with those cytochromes which have a redox midpotential high enough to activate oxygen molecules. In this communication, evidence is presented for the interaction of BW755C with cytochrome oxidase of mitochondria and for the inhibition by BW755C and PPDA of the production of active oxygen.

Materials and methods. Reagents. BW755C and pure beef heart cytochrome oxidase were kindly supplied by Dr M. Hori, Gifu College of Pharmacy, and Dr Y. Orii, Kyoto University, respectively. Tris, ADP, and zymosan A were obtained from Sigma Chem. Co. Luminol was a product of Wako Chemicals. Bovine serum albumin was purchased from Armour Pharm. Co. Other reagents were of the purest grade commercially available.

Preparation of rat liver mitochondria and electron transport particles (ETP) and measurement of oxygen uptake by mitochondria. Rat liver mitochondria were prepared essentially by the method of Schneider[12] using 0.25 M sucrose which contain 0.5 mM EDTA and 10 mM Tris-Cl (pH 7.4). Electron transport particles (ETP) were prepared from rat liver mitochondria according to the procedure of Ruzicka and Crane[13]. Oxygen up-

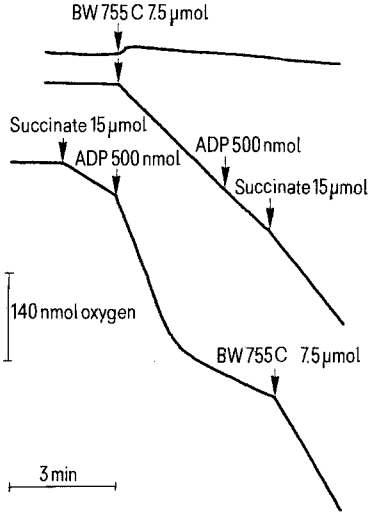

Figure 1. Effect of BW755C on mitochondrial respiration. The reaction mixture was composed of 675 µmol sucrose, 30 µmol KCl, 15 µmol $MgCl_2$, 15 µmol phosphate, 1.5 µmol EDTA, 60 µmol Tris-Cl, and 3 mg of mitochondrial protein in a final volume of 3 ml (pH 7.4). Reaction was carried out at 30 °C. For curve 1 no mitochondria were present.

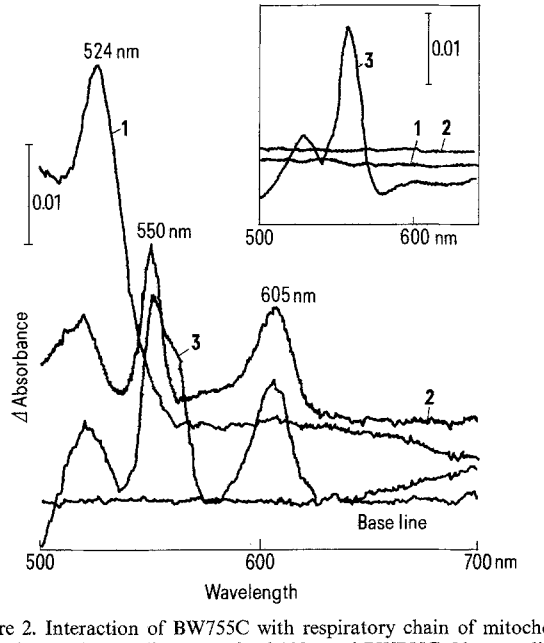

Figure 2. Interaction of BW755C with respiratory chain of mitochondria. The reaction medium contained 100 nmol BW755C, 20 mg sodium cholate, 500 µmol sucrose, 100 µmol Tris-Cl, and 3.7 mg of ETP protein in a final volume of 2.0 ml (pH 7.8). Anaerobic condition was obtained using N_2 gas. The insert shows the experiment with microsomes instead of mitochondria. Reaction was carried out at 24 °C.

take by mitochondria was measured by a Galvani-type oxygen electrode (Sensanics Japan Co.). The redox reaction between BW755C and cytochromes in ETP was spectrophotometrically measured using a Hitachi 320S recording spectrophotometer.

Preparation of rat liver microsomes. Rat liver microsomes were prepared from the post mitochondrial supernatant fraction by centrifugation at 105,000 × g for 60 min and were twice washed by centrifugation with the same medium as that used for the preparation of mitochondria. Microsomes thus prepared did not contain any spectrophotometrically detectable cytochrome oxidase of mitochondria. Protein content was determined by the method of Lowry et al. using bovine serum albumin as a standard protein[14].

Detection of active oxygen in macrophages. Peritoneal exudate cells (macrophages) were collected using 60 ml of sterilized MEM (Nissui Seiyaku Co.) from the peritoneal cavity of rats weighing 200–250 g. The animals had been injected i.p. with 10 ml of paraffin oil four days earlier. The collected cells were twice washed in sterilized MEM by centrifugation at 80 × g for 5 min. Active oxygen in macrophages (10^6 cells/0.5 ml) was assayed by using a photon counter (Berthold LB 9500T) detecting the chemiluminescence due to the oxidation of luminol, as previously described in detail[15].

Results and discussion. The effect of BW755C on oxidative phosphorylation of isolated rat liver mitochondria. Figure 1 depicts the oxygram of mitochondrial respiration measured by using a Galvani-type oxygen electrode. Isolated mitochondria showed tightly coupled respiration exhibiting a high respiratory control index[16]. BW755C exerted no significant influence on oxidative phosphorylation of mitochondria at concentrations lower than 200 μM (data not shown), whereas many uncoupling agents containing several NSAIDs strongly perturb mitochondrial respiration[17–19]. However, at high concentrations, BW755C quickly released the state 4 respiration (a respiration depressed for lack of ADP) like the uncoupling agents (curve 3). Furthermore BW755C induced oxygen uptake by mitochondria without added substrate for the respiratory chain (curve 2), indicating an effect of BW755C unlike those of typical uncoupling agents. The uncoupling agents, which work as protonophores, release the state 4 respiration in the presence of substrate for the respiratory chain. BW755C alone did not consume any oxygen without mitochondria (curve 1). The oxygen uptake by ETP was also initiated by the drug without added substrate and the K_m value of BW755C for oxygen uptake by ETP was about 1.5 mM (data not shown). These findings apparently demonstrate that BW755C is able to supply electrons to the mitochondrial respiratory chain.

The interaction of BW755C with the respiratory chain in mitochondria. The interaction of BW755C with the respiratory chain of mitochondria was spectrophotometrically examined for ETP. Figure 2 shows the difference spectra of ETP with and without BW755C under anaerobic conditions. The difference spectrum measured immediately after addition of BW755C to the sample gave an absorption maximum at 524 nm (curve 1) and the maximal peak quickly disappeared in the course of incubation for 10 min. Curve 2 depicts the absorption curve measured after incubation for 30 min, where the absorption maximum at 524 nm was no longer detected and a typical reduced minus oxidized difference spectrum of cytochromes *c* and aa_3 was observed, with absorption maxima at 550 and 605 nm. When the sample was treated with dithionite, an absorption shoulder, which is ascribed to the reduction of cytochrome *b*, was seen at 560 nm in addition to the absorption maxima at 550 and 605 nm (curve 3). These results may indicate that BW755C is able to give electrons to cytochrome *c* and aa_3 but not to cytochrome *b*. The brief appearance of the absorption maximum at 524 nm suggests the production of an unstable oxidized intermediate of BW755C. The insert in figure 2 shows the results obtained with microsomes; curves 2 and 3 express the difference spectra of microsomes treated with BW755C and dithionite, respectively. BW755C did not undergo interaction with cytochrome *b* in microsomes.

Evidence for the direct interaction between BW755C and cytochrome oxidase was presented by using cytochrome oxidase purified from beef heart mitochondria (fig. 3). Curve 1 shows the difference spectrum of cytochrome oxidase treated with dithionite, where a typical absorption spectrum of cytochrome oxidase was seen at 605 nm. The addition of BW755C instead of dithionite gave the typical reduced minus oxidized difference spectrum of cytochrome oxidase (curve 2). NSAIDs such as indomethacin, benoxaprofen, flufenamic acid, and tinoridine did not interact with cytochromes in mitochondria but caused significant depression of mitochondrial respiration[20], indicating a mode of action of BW 755C on the respiratory chan in mitochondria different from those of these NSAIDs.

The inhibitory effects of BW755C and PPDA on the active oxygen-generating system in macrophages. BW755C, which has been known to inhibit the production of active oxygen[15], was found to interact with cytochrome oxidase as mentioned above. It is of much interest to see whether PPDA, which interacts with cytochrome oxidase of mitochondria[8], depresses the generation of active oxygen in phagocytes. Indeed, as shown in figure 4, PPDA inhibited the production of active oxygen in the macrophage, exhibiting a stronger effect than that of BW755C. These findings strongly support the correlation between its interaction with cytochrome(s) in mitochondria and its potent inhibitory effect on the formation of oxygen

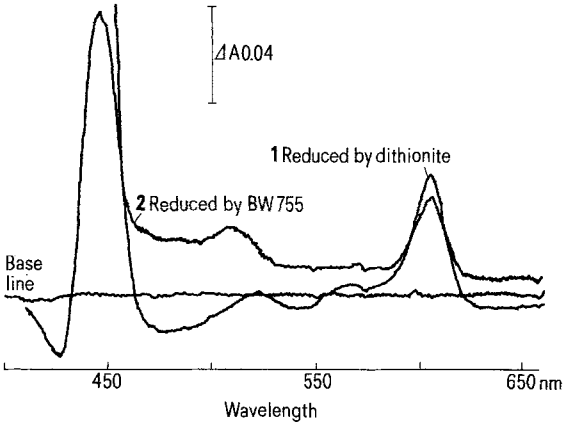

Figure 3. Interaction of BW755C with cytochrome oxidase. The reaction conditions were the same as in figure 2. 7.5 μmol cytochrome oxidase from beef heart mitochondria was present instead of ETP.

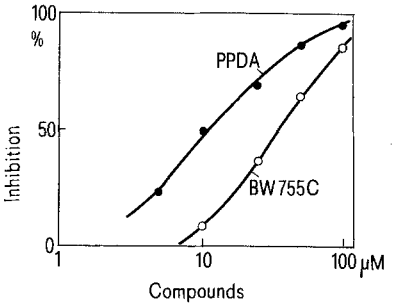

Figure 4. Effects of BW755C and PPDA on active oxygen-generating system in macrophages. The reaction mixture was composed of 10^6 cells of macrophages and 200 μg of luminol in 0.5 ml MEM, containing 1% rat plasma. The reaction was initiated by adding zymosan A suspension and was performed at 37 °C.

radicals. The involvement of cytochrome *b* in the active oxygen-generating system has also been proposed[9-11], but it is not known whether cytochrome *b* activates oxygen molecules by forming oxygenated cytochrome *b*, as in cytochrome oxidase[21,22]. If the redox midpotential of the cytochrome *b* were nearly as high as that of cytochrome oxidase or the steric factor of the cytochrome *b* permitted, BW755C and PPDA would interact with the cytochrome *b*. There is another possibility for their pharmacological actions; these drugs could be activated by mitochondrial cytochrome oxidase to act as radical scavengers. This may be supported by the short-termed appearance of a compound possessing an absorption maximum at 524 nm. For the better understanding of the molecular mechanism of the potent inhibition of BW755C on the cyclo- and lipo-oxygenases in macrophages, more extensive studies with macrophages are needed; such studies are currently being carried out in our laboratory.

Abbreviations. PPDA, p-phenylenediamine; NSAIDS, non-steroidal anti-inflammatory drugs; MEM, minimum essential medium.

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5 Ferreira, S.H., and Vane, J.R., in: The Prostaglandins 2, p.1. Ed. P.W. Ramwell. Plenum Press, New York 1974.
6 Moncada, S., and Vane, J.R., in: Advances in Internal Medicine, p.1. Ed. G.H. Stollerman. Year Book Medical Publishers, Chicago 1979.
7 Higgs, G.A., Flower, R.J., and Vane, J.R., Biochem. Pharmac. *28* (1979) 1959.
8 Orii, Y., and Okunuki, K., J. Biochem. *53* (1963) 489.
9 Segal, A.W., and Jones, O.T.G., Biochem. biophys. Res. Commun. *88* (1979) 130.
10 Gabig, T.G., Schervish, E.W., and Santinga, J.T., J. biol. Chem. *257* (1982) 4114.
11 Gabig, T.G., and Lefker, B.A., Biochem. biophys. Res. Commun. *118* (1984) 430.
12 Schneider, W.C., J. biol. Chem. *176* (1948) 259.
13 Ruzicka, F.J., and Crane, F.L., Biochim. biophys. Acta *223* (1970) 71.
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
15 Mibu, H., Hasegawa, J., Niwa, M., Nozaki, M., Tsurumi, K., and Fujimura, H., Folia pharmac. Japan *83* (1984) 355.
16 Chance, B., and Williams, G.R., J. biol. Chem. *227* (1955) 383.
17 Brody, T.M., Pharmac. Rev. *7* (1955) 335.
18 Terada, H., Biochim. biophys. Acta *639* (1981) 225.
19 Heytler, P.G., in: Inhibitors of Mitochondrial Function, p.199. Eds M. Erecinska and D.F. Wilson. Pergamon Press, Oxford/New York 1981.
20 Shiojiri, H., Kawai, K., Nozawa, Y., Nozaki, M., Tsurumi, K., and Fujimura, H., Presentation at Ist World Conference on Inflammation (Italy) 1984.
21 Orii, Y., and Okunuki, K., J. Biochem. *53* (1963) 489.
22 Orii, Y., and Okunuki, K., J. Biochem. *57* (1965) 45.

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Androstenedione or corticosterone treatment during pregnancy alters estrous cycle of adult female offspring in mice

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Summary. Female offspring from mice injected with androstenedione during late pregnancy showed lengthened vaginal cycles, persistent estrus and decreased incidence of pro-estrus and diestrus, whilst offspring from mice injected with corticosterone showed increased incidence of diestrus. These observations give qualified support to the hypothesis that stress during pregnancy alters the female offspring reproductive system through the action of adrenal steroids.
Key words. Mouse, female offspring; androstenedione treatment; corticosterone treatment; pregnancy; estrous cycle.

The effects of stress during pregnancy in reducing sexual behavior of the male offspring in rodents is well known[1-3]. Ward[1] and Dahlöf et al.[2] have suggested that this effect is mediated by increased maternal pituitary-adrenocortical output and that in utero exposure to adrenal steroids compromises sexual-behavioral differentiation of the fetal male: we have recently contributed evidence supporting this hypothesis[3]. Similarly, evidence exists to suggest that stress during pregnancy influences sexual development of female offspring: in rats, prenatal stress lengthens the estrus-metestrus stages of the adult cycle[4], whilst in mice prenatal stress shortens the length of the pro-estrus stage and delays puberty[5]. No experiments to date have tested the hypothesis that the effects of stress during pregnancy upon female offspring reproductive function are mediated by in utero exposure to maternal adrenocortical products. In rats and mice the major adrenocortical product released in the stress response is corticosterone[6], but androgens, estrogens and progestagens can also be secreted from the adrenal cortex under the influence of ACTH[7,8]. Stress during pregnancy increases maternal and fetal plasma androstenedione concentrations in the rat[9], and it has been suggested that fetal exposure to this compound disrupts sexual differentiation[1,9]. Additionally, as corticosterone[10] and most other unbound steroids cross the placenta[11], it is possible that fetal exposure to other adrenocortical products may mediate the effects of stress during pregnancy upon the female offspring. The purpose of this study was to examine the effects of androstenedione or corticosterone administration during late pregnancy, upon the estrous cycle of adult female offspring.

Materials and methods. Females used were virgin TO mice (A. Tuck and Sons, Battlesbridge, Essex), obtained two weeks prior to mating. They were housed in groups of 10 in large plastic cages (42 × 25 × 11 cm), allowed ad libitum supply of food (Labsure animal diet, Christopher Hill Ltd, Dorset) and water, and maintained on a reverse lighting regime (red lights on 12.00–22.00 h) at 18–23 °C. At 10–12 weeks of age, these females were placed individually into small plastic cages (30 × 13 × 11 cm) with a male and observed daily for the appearance of a vaginal plug, which was deemed to indicate day 0 of pregnancy. Males were then removed and females were left undisturbed until day 12 of pregnancy when treatments were administered. Pregnant females were randomly assigned to 1 of 4 treatments: untreated controls (n = 8), steroid vehicle injection controls (n = 7), corticosterone treatment (n = 8) and