Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat inflammation and pain. In the present study, we examined the effects of celecoxib, a cyclooxygenase-2 (COX-2)-selective NSAID, on rat liver mitochondrial function. Celecoxib dose-dependently induced mitochondria swelling, which was not suppressed by cyclosporine A (CsA). The oxygen consumption rate in mitochondria-suspended solution was facilitated by the addition of celecoxib, and its uncoupling activity was observed. Celecoxib also suppressed SF6847-induced uncoupling, and appeared to exert inhibitory effects on the electron transport chain. Celecoxib suppressed the state 3 oxygen consumption rate in the presence of ADP. Protein release from the mitochondrial matrix was detected following the addition of celecoxib, and aldehyde dehydrogenase 2 (ALDH2) and hydroxymethylglutaryl-CoA (HMG-CoA) synthase 2 (HMGCS2) bands were confirmed in a Western blot analysis. On the other hand, protein release of cytochrome C (CytC), which is an inducer of apoptosis, from the intermembrane space was not observed. Celecoxib enhanced the membrane permeability of human erythrocytes and synthesized liposomes dose-dependently. It then induced the membrane-involving mitochondrial swelling and suppressed mitochondrial function.

Key words mitochondrial permeability transition; celecoxib; membrane

MATERIALS AND METHODS

Reagents Celecoxib was purchased from LC Laboratories (Woburn, MA, U.S.A.). Mefenamic acid, fenbufen were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Naproxen, etodolac, salicylic acid, loxoprofen, acetylsalicylic acid, indomethacin, ibuprofen, piroxicam, and meloxicam were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Celecoxib, naproxen, etodolac, salicylic acid, loxoprofen, acetylsalicylic acid, indomethacin, and ibuprofen were dissolved in ethanol and stored at −20°C until use. Mefenamic acid, fenbufen, piroxicam, and meloxicam were dissolved in dimethyl sulfoxide and stored at −20°C until use. SF6847 (sc-200569) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Cyclosporine A (CsA) was purchased from Nacalai Tesque, Inc. Anti-voltage dependent anion channel (VDAC) antibody were purchased from Cell Signaling Technology Japan (Tokyo, Japan). Anti-cytochrome C (CytC) antibody and anti-adenylate kinase 2 (AK2) antibody were purchased from Signalway Antibody (Pearland, TX, U.S.A.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), respectively. Anti-aldehyde dehydrogenase 2 (ALDH2) antibody was purchased from Abcam (Cambridge, U.K.). Anti-hydroxymethylglutaryl-CoA (HMG-CoA) synthase 2 (HMGCS2) antibody and anti-adenine nucleotide translocator 1 (ANT) antibody were kindly gifted from Professor Yasuo Shinozuka (Tokushima University, Tokushima, Japan). Anti rabbit immunoglobulin G (IgG) of donkey origin (secondary antibody) was purchased from GE Healthcare Japan

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Preparation of Rat Liver Mitochondria Preparation of liver mitochondria were performed as previously described from male Wistar rats. Protein concentration of prepared mitochondrial suspension was examined by the Bicinchoninic acid method using bovine serum albumin as standard. All animal treatment was based on the animal care regulations of Suzuka University of Medical Science.

Swelling Analysis of Mitochondria Absorbance decreasing of mitochondrial suspension at 540 nm was examined at 25°C by spectrophotometric analysis using UV-1800 spectrophotometer (Shimadzu Co., Kyoto, Japan). Mitochondria were incubated in inorganic phosphate (Pi)-containing medium (+Pi medium: sucrose (200 mM), K/Pi buffer (10 mM), pH 7.4) at 0.7 mg protein/mL of final concentration, and energized by succinate (10 mM) and rotenone (1 μg/mL). NSAIDs or Ca²⁺ (100 μM) were added to the mitochondrial suspension, and the time-dependent absorbance change at 540 nm was examined.

Analysis of Mitochondrial Function Oxygen consumption rates (e.g. state4, state3, uncoupling) of mitochondria were examined using the +Pi medium as previously described. Oxygen consumption rate was analyzed with a Clark type oxygen electrode (Yellow Spring 5331) as previously described method.

Analysis of Protein Release from Mitochondrial Compartments Mitochondria were incubated with 200 μM celecoxib (10 min, 25°C). After celecoxib treatment, the incubated solution were centrifuged (13200 rpm, 4°C, 5 min), and obtained the supernatant and precipitation. To evaluate the celecoxib-induced protein release from mitochondria, we subjected the mitochondrial lysates (10.5 mg) to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western analysis by using the first antibodies (anti-VDAC, -CytC, -AK2, -ALDH2, -HMGCS2, and -ANT antibody). SDS-PAGE on 12.5 or 15.0% gel was performed as described by Laemmli. Sample proteins that were obtained as described in the above were dissolved in 25 mM Tris–HCl (pH 8.0) with centrifugation. Washed erythrocytes were centrifuged and the supernatant at 540 nm was assessed using the microplate reader Infinite M200 (TECAN, Mannedorf, Zurich, Switzerland). Hemolytic activity was calculated as previously described.

Calcein-loading Large Unilamellar Vesicles (Cal-LUVs) were prepared according to previously described methods. A total of 7.6 μg of POPC was dissolved in chloroform and dried by a nitrogen gas stream. Any remaining chloroform was completely removed under a vacuum for 2 h. The lipid membrane that formed was hydrated with 1 mL calcein solution (60 mM caeline, 200 mM sucrose, and 10 mM KCl, pH 7.4) for 30 min. The hydrate became multilamellar vesicles (MLV) by vortexing. In order to prepare LUVs, MLV suspensions were extruded 11 times through a 100-nm polycarbonate membrane filter using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, U.S.A.) at 80°C. Cal-LUV suspensions were passed through a Sephadex-G25 column twice to remove extravesicular calcein. The quantitative determination of prepared Cal-LUV was performed by the quantification of the phospholipid contents using LabAssay Phospholipid kit (Wako Pure Chemical Industries, Ltd.). Cal-LUVs were suspended in +Pi medium with the compound being tested (e.g. celecoxib) at 25°C for 60 min, and 200 μL of the suspension was added to a fluorescence assay black plate. Calcein release from Cal-LUVs was evaluated by fluorescence at 490 nm (Excitation) and 520 nm (Emission) using Gemini X-ray photoelectron spectroscopy (XPS) (Molecular Devices LLC., Sunnyvale, CA, U.S.A.).

RESULTS

NSAIDs Induced Mitochondrial Swelling The ability of various NSAIDs to induce Ca²⁺-like mitochondrial swelling was examined (Table 1). Celecoxib (200 μM) induced similar mitochondrial swelling to Ca²⁺ (100 μM), and a decrease in absorbance (ΔAbs₅₄₀) of 96.1% was observed. Mefenamic acid (200 μM) also induced swelling and ΔAbs₅₄₀ was 72.7%.

| NSAIDs       | ΔAbs₅₄₀ (%) |
|--------------|------------|
| Ca²⁺         | 100.0      |
| Celecoxib    | 96.1±7.7   |
| Mefenamic acid| 72.7±20.1  |
| Fenbufen     | 26.6±16.3  |
| Diclofenac   | 15.3±10.3  |
| Etodolac     | 9.1±9.4    |
| Salicylic acid| 8.7±4.8    |
| Loxoprofen   | 5.1±5.7    |
| Naproxen     | 11.2±4.8   |
| Acetylsalicylic acid | 4.3±0.3   |
| Indomethacin | 6.7±1.3    |
| Ibuprofen    | 6.4±4.2    |
| Piroxicam    | 0.1±5.0    |
| Meloxicam    | 0.1±6.4    |

Mitochondrial swelling was examined as absorbance decrease at 540 nm (ΔAbs₅₄₀). After Ca²⁺ (100 μM) addition, the ΔAbs₅₄₀ during 10 min are shown as 100%. Each drug (200 μM) was added to mitochondria, and the ΔAbs₅₄₀ during 10 min was determined. The results are shown as the mean± standard deviation (S.D.) obtained from 3 independent experiments.
Other NSAIDs (200 µM) tested in the present study exhibited a weak ability to induce mitochondrial swelling. Fenbufen (26.6%), diclofenac (15.3%), and naproxen (11.2%) induced swelling. Etodolac, salicylic acid, loxoprofen, acetylsalicylic acid, indomethacin, and ibuprofen weakly induced swelling (5.1−9.1%), while that induced by piroxicam and meloxicam was negligible.

The structure–activity relationship between mefenamic acid derivatives and mitochondrial function has already been reported. In the present study, we examined the effects of celecoxib on mitochondrial function in more detail.

**Effects of Celecoxib on Mitochondrial Swelling Pi**

Pi is an essential element for calcium-induced mPT, and Ca^{2+} (100 µM) addition to mitochondrial suspensions induced the mitochondrial swelling, and decreased absorbance at 540 nm (solid line in Fig. 1A). In the absence of Pi, Ca^{2+} addition did not markedly affect changes in absorbance (dashed line in Fig. 1A). CsA (1 µM) suppressed Ca^{2+}-induced absorbance change completely (dotted line in Fig. 1A). Celecoxib (20–200 µM) dose-dependently decreased absorbance at 540 nm (Fig. 1B). Celecoxib-induced decrease in absorbance were not suppressed by CsA (1 µM) addition (dotted line in Fig. 1B). Thus, celecoxib exhibited discriminative feature for the modification of mitochondrial membranes.

**Effects of Celecoxib on Mitochondrial Function**

Since the de-energization of mitochondria (e.g. uncoupling) had been shown to facilitate the mPT induction, we examined the effects of Ca^{2+} and celecoxib on the oxygen consumption in mitochondria. The addition of Ca^{2+} (100 µM) induced uncoupling, and oxygen consumption increased markedly (Fig. 2A). This Ca^{2+}-induced uncoupling was completely suppressed by the addition of CsA (1 µM) (Fig. 2A). In order to avoid the promotion of oxygen consumption by mPT, mitochondria were pretreated with CsA (1 µM), and un-
coupling states were examined. The rate of basal oxygen consumption (state 4) was 32.3 natomsO/mg/min (0 µM celecoxib line in Fig. 2B), and increased to 151.6 natomsO/mg/min with SF6847 (100 nM, positive control of uncoupling) (Fig. 2B). Celecoxib dose-dependently increased the oxygen consumption rate (6.3–200 µM) (Fig. 2B). The oxygen consumption rate with 200 µM celecoxib was 96.8 natomsO/mg/min, which was 54.0% of SF6847-induced uncoupling rate. Moreover, celecoxib inhibited SF6847-induced uncoupling, and the oxygen consumption rate decreased by 34.2 and 78.1% following the addition of 100 and 200 µM celecoxib, respectively (Fig. 2C). These results indicate that celecoxib affects the mitochondrial electron transport system.

Energy synthesis is one of the key mitochondrial functions, and the state 3 rate is an index of ATP synthesis in mitochondria. Therefore, the inhibition ratio of celecoxib to the state 3 oxygen consumption rate was examined. As shown in Fig. 3, celecoxib dose-dependently inhibited the state 3 rate. The state 3 rate was inhibited by 41.7% following the addition of 200 µM celecoxib.

**Effects of Celecoxib on Protein Release from Mitochondrial Compartments** Celecoxib concentrations higher than 50 µM increased protein release from mitochondria, and protein bands were detected using an SDS-PAGE analysis of supernatants (lanes 8–10 in Fig. 4B). Thus, mitochondrial component proteins were released by celecoxib-induced swelling.

VDAC (outer-membrane marker protein) was not released by celecoxib (200 µM) and the VDAC band was only detected with precipitation (lane 3 in Fig. 4C). CytC and AK2, intermembrane space marker proteins, were not detected in the supernatant following the addition of celecoxib (lane 3 in Fig. 4D), whereas ALDH2 and HMGC2, matrix marker proteins, were present (lane 3 in Fig. 4D). HMGC2 was fully released from the matrix compartment, and no bands were observed with precipitation (lane 3 in Fig. 4C). The release of ANT (an inner-membrane marker protein) was not detected following the addition of celecoxib (lane 3 in Fig. 4D).

Ca²⁺, an inducer of mitochondrial swelling, released CytC, AK2, ALDH2, and HMGC2 at a concentration of 100 µM, and these bands were detected in the supernatant (lane 2 in Fig. 4D). Ca²⁺ released intermembrane space proteins (CytC and AK2) and the matrix proteins (ALDH2 and HMGC2).

**Effects of Celecoxib on Lipid Membranes** The modifying effects of celecoxib on the permeability of lipid membranes were examined by a hemolytic analysis of human erythrocytes. Hemolysis was detected following the addition of celecoxib, with hemolysis ratios of 4.0% (50 µM), 55.8% (100 µM), and 93.6% (200 µM) being observed (Table 2).

In the permeability analysis of calcein including POPC-liposomes, calcein release was detected following the addition of celecoxib. Calcein release ratios of 23.7, 79.3, and 90.1% were observed following the addition of 50, 100, and 200 µM celecoxib, respectively (Table 2).

**DISCUSSION**

NSAIDs are used as anti-inflammatory and anodyne medicine, but are associated with several adverse effects such as hepatopathy and gastrointestinal tract disturbances. A loss of liver mitochondrial function is considered as a factor of hepatopathy. Ca²⁺ induces mitochondrial swelling by formation of mitochondrial permeability transition (mPT) pore, and injures the mitochondrial function. SF6847 disappears the H⁺ gradient in mitochondrial inner membrane, and obstructs the mitochondrial function. We examined the effect of NSAIDs on mitochondria, and observed celecoxib-induced mitochondrial swelling (Table 1). The state of mitochondrial membrane proteins, which participate in the mitochondrial electron transport system, is altered by the mitochondrial swelling. Mitochondrial swelling leads to liver dysfunction.

A number of NSAIDs (e.g. meclofenamic acid, tolfenamic acid, flufenamic acid, diclofenac, and N-phenylanthranilic acid) have been shown to dose-dependently induce mitochondrial swelling. Their induction of mitochondrial swelling was suppressed by CsA (1 µM). Various NSAIDs (e.g. 200 µM piroxicam, meloxicam in Table 1) exhibited no swelling effect on mitochondria, and it is difficult to think that mitochondrial swelling depends only on the osmotic pressure of NSAID. In the present study, celecoxib-induced mitochondrial swelling occurred in a dose-dependent manner up to 200 µM and was not suppressed by CsA (Fig. 1). These results indicate that celecoxib-induced swelling involves a different mechanism to that of calcium-induced swelling. Induction effect of mitochondrial swelling by mefenamic acid has been reported, and the structure of pharmacophore, which participates in mitochondrial function, was previously reported.

A relation between the activity balance of COX-1 and COX-2 and the side effect of NSAIDs (e.g. hepatopathy and gastroenteropathy) have been examined. Since gastroenteropathy occur when COX-1 is strongly obstructed, medication that selectively obstructs COX-2 has been developed. COX-2-selective celecoxib dose-dependently exerts uncoupling effects on mitochondria, similar to SF6847 (Figs. 2A, B). However, celecoxib-induced uncoupling is not suppressed by CsA, and, thus, celecoxib modifies mitochondrial function via a different mechanism to Ca²⁺. Moreover, celecoxib dose-dependently restrained the uncoupling effects of SF6847, which suggests that it also obstructs the electron transfer.

**Fig. 3. Inhibition Effect of Celecoxib on the State 3 Oxygen Consumption**

Mitochondria were suspended (0.7 mg/mL of protein) in +Pi medium with succinate (10mM) and rotenone (1 µM) as respiratory substrates, and state 3 oxygen consumption rate was examined with ADP (1mM). Effect of celecoxib (0–200 µM) on the oxygen consumption in ADP coexistent medium was monitored during 10minutes as well as Fig. 2. The results of state 3 inhibition (%) are shown as the mean±S.D. obtained from 3 independent experiments.
system of mitochondria (Fig. 2C). SF6847 disappears the H\(^+\) gradient of mitochondrial inner membrane and indicates the biggest oxygen consumption rate. This oxygen consumption is including the oxygen consumption by an electron transfer system. An electron transfer system was obstructed by celecoxib, and SF6847 induced oxygen consumption rate decreased by celecoxib addition. Celecoxib inhibited state 3, which involves ATP synthesis in the mitochondrial inner membrane (Fig. 3), and, thus, may suppress mitochondrial function. Celecoxib has been suggested to modify membrane conditions (e.g. fluidity), and exert at least 2 of the following effects: 1; the induction of uncoupling actions through a change in the membrane permeability of protons, and 2; the induction of irregularities in the electron transfer system through functional modifications to inner membrane-localized proteins involved in electron transfer. Since a decrease in absorbance (mitochondrial swelling) was not observed with SF6847 (data not shown), celecoxib appears to use a different mechanism of action to SF6847.

Celecoxib-induced protein release from mitochondrial compartment (the outer membrane, inner membrane, intermembrane space, and matrix) was also investigated in the present study, and the isolation of matrix proteins (e.g. ALDH2 and HMGCS2) was confirmed (Fig. 4). The degree of release of these celecoxib-induced matrix proteins was greater than

Table 2. Effect of Celecoxib on the Permeability of Lipid Membrane

| Celecoxib (µM) | Erythrocyte (%) | POPC-liposome (%) |
|----------------|-----------------|-------------------|
| 0             | 0.0±0.0         | 0.0±0.0           |
| 50            | 4.0±0.7         | 23.7±1.5          |
| 100           | 55.8±3.4        | 79.3±4.6          |
| 200           | 93.6±1.9        | 90.1±4.3          |

The 100% lysis (control) of tested membrane was obtained by using de-ionized water (erythrocyte) and 1% triton X-100 (POPC-liposome). The membrane lysis ratio (%) to control was determined. Results are shown as the mean±S.D. obtained from 3 independent experiments.
calcium-induced protein release, indicating that mitochondrial morphology is strongly modified (e.g. aggregation) by the addition of celecoxib. The release of marker proteins from the outer membrane, inner membrane, and intermembrane space was not observed following the addition of celecoxib. Celecoxib produced a unique permeability system that differed from calcium-induced mPT. The release of CytC was not observed following the addition of celecoxib, suggesting that celecoxib did not induce Ca\(^{2+}\)-like apoptosis.

Celecoxib caused hemolysis in a dose-dependent manner, with 93.6% of erythrocytes being lysed by the addition of 200\(\mu\)M celecoxib (Table 2). Celecoxib also induced the lysis of POPC-liposomes, with 90% of liposomes being lysed by 200\(\mu\)M celecoxib. Celecoxib appears to recognize the general constituents of membranes (e.g. phospholipid) and modifies membrane features (e.g. fluidity). Mitochondrial membranes (particularly the inner membrane) contain many types of proteins (e.g. electron transporters and substrate transporters), and differ physically from general phospholipid cell membranes. After the binding of celecoxib to the mitochondrial membrane, the mitochondrial structure seemed to be maintained temporarily. However, the matrix proteins may have been released through the opening of the mPT pore-like system. The effects of pharmaceuticals, which cause hepatopathy as a side effect, on mitochondrial function are being investigated. The relation between COX-1, COX-2 selectivity and mitochondrial dysfunction (e.g. swelling) is the subject of future investigation. The analysis of mitochondrial function may not be related to a clinical event directly, but the mitochondria associated examination (e.g. NSAID induced swelling) should be useful for understanding the feature of drugs.

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Conflict of Interest The authors declare no conflict of interest.

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