Effect of \(N\)-Phenylanthranilic Acid Scaffold Nonsteroidal Anti-inflammatory Drugs on the Mitochondrial Permeability Transition

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Received September 18, 2015; accepted November 13, 2015

Hepatotoxicity is a known side effect of nonsteroidal anti-inflammatory drugs (NSAIDs). In the present study, the effects of \(N\)-phenylanthranilic acid (NPA) scaffold NSAIDs on rat liver mitochondria were examined. Mefenamic acid (MEF, 200 \(\mu\)M) induced mitochondrial swelling, which was inorganic phosphate (Pi)-dependent and suppressed by cyclosporin A (CsA, 2.5 \(\mu\)M), similar to calcium-induced swelling. Mitochondrial swelling was also observed following the addition of 200 \(\mu\)M flufenamic acid (FLU), meclofenamic acid (MCL), and tolfenamic acid (TOL). Less swelling was observed with the addition of 200 \(\mu\)M diclofenac (DIC) or NPA. Diphenylamine (DPA)-induced swelling occurred in a Pi-independent manner and was not sensitive to CsA. The mechanism by which DPA interacted with the mitochondrial inner membrane differed from those of the other NPA scaffold NSAIDs. The addition of 50 \(\mu\)M MEF, MCL, TOL, and FLU had uncoupling effects in mitochondrial inner membrane. These NSAIDs dose-dependently obstructed electron transport in the respiratory chain. NSAIDs are known to have various dynamic structures, and the solvation free energies (dGWs: an index of stereo-hydrophobicity) of the conformers obtained were determined using a molecular orbital analysis. The relationship between the dynamic structures and swelling induced by NPA scaffold NSAIDs was also examined.

Key words nonsteroidal anti-inflammatory drug; mitochondrial permeability transition; diphenylamine; hydrophobicity

The mitochondrion is a cellular organelle that is responsible for the production of ATP in eukaryotic cells. The inner mitochondrial membrane is maintained in a highly impermeable state in order to prevent most substance, even solutes and ions, passing through into the mitochondrial matrix. However, the permeability of inner membrane markedly increases under certain conditions, such as in the presence of Ca\(^{2+}\) and inorganic phosphate (Pi). This change in permeability is known as the mitochondrial membrane permeability transition (mPT).\(^1\) In mPT, open ion channel complexes transport solutes and ions with a mass of up to approximately 1.5 kDa.\(^1,3\) When mPT is induced, mitochondria swell and the membrane potential dissipates, resulting in the release of various inner membrane proteins (e.g., cytochrome c). This mPT-related protein release subsequently triggers apoptosis and necrosis.\(^4,5\)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of number of disorders associated with pain and inflammatory because they produce antipyretic and analgesic action by inhibition of cyclooxygenase (COX) and decreasing prostaglandin levels.\(^6,7\) Although rare, the adverse side effects associated with NSAIDs include gastric ulceration and damage to the liver and kidneys. These side effects have been attributed to the inhibition of COX activity, with several studies implicating a COX-dependent mechanism.\(^8\)–\(^10\) Mitochondria have been the subject of recent research as a principal target of NSAID-induced toxicity.\(^11\)–\(^16\)

Fenamic acid is an aromatic amino acid that is also called \(N\)-phenylanthranilic acid (NPA). NSAIDs such as mefenamic acid (MEF), meclofenamic acid (MCL), tolfenamic acid (TOL), and flufenamic acid (FLU) are derived from fenamic acid, and are members of the fenamic acid family (Fig.1).

Since diphenylamine (DPA) is similar in structure to fenamic acid, we herein examined the effect of NPA scaffold NSAIDs, such as MEF, MCL, TOL, FLU, diclofenac (DIC), and DPA on mPT, which is involved in functional regulation.

MATERIALS AND METHODS

Reagents MEF was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). MCL, TOL, FLU, DIC, NPA and DPA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Stock solutions of MEF and its related compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at \(-20^\circ\text{C}\) until use. Cyclosporine A (CsA) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). SF6847 (sc-200569) was purchased from Santa Cruz Biotechnology, Inc. (CA, U.S.A.).

Preparation of Mitochondria Mitochondrial suspensions were prepared from the livers of male Wistar rats according to previously described methods.\(^17,18\) The protein concentrations of mitochondrial suspensions were determined using a Biuret analysis with bovine serum albumin as the standard. All animal experiments were conducted according to the animal care regulations of Suzuka University of Medical Science.

Measurements of Mitochondrial Swelling Decreases in the absorbance of mitochondrial suspensions at 540 nm (an index of mitochondrial swelling) were determined at 25°C using UV-1800 spectrophotometer (Shimadzu Co., Kyoto, Japan) as previously described.\(^19,20\) Rat liver mitochondria were incubated in inorganic Pi-containing medium (\(+\)Pi medium; 200 mM sucrose, 10 mM K/Pi buffer, pH 7.4) at a final protein concentration of 0.7 mg protein/mL, and energized by 10 mM succinate and 1 \(\mu\)g/mL rotenone. The NSAIDs being...
tested or 100 µM Ca2+ were added to the suspension, and time-
dependent changes in absorbance were monitored.

**Examination of Mitochondrial Oxygen Consumption**

Mitochondrial oxygen consumption and uncoupling rates were examined using the +Pi medium described in the Measurement of Mitochondrial Swelling section. The dissolved oxygen concentration in the medium was determined using a Clark oxygen electrode (Yellow Spring 5331) according to a previously reported method.18)

**Molecular Analysis of NPA Scaffold NSAIDs**

The initial structures of the NSAIDs being tested (MEF, MCL, TOL, FUL, DIC, NPA, and DPA) were constructed using CAChe (Fujitsu Ltd., Tokyo, Japan). A conformation analysis of NPA derivatives was performed using CONFLEX with a MM2 forcefield (Fujitsu Ltd.).21,22) Heat formation energy calculations of the conformers were performed with parametric method 3 (PM3) Hamiltonian using the MOPAC program (Fujitsu Ltd.). The stable and transient structures of NPA derivatives were initially constructed using the general parameters of the bond and dihedral angle, and were then refined using eigenvector following (EF) optimization methods. The solvation free energy (an index of stereo-hydrophobicity) was determined for all conformers obtained using the MOPAC parameter as previously reported.23–25)

**RESULTS**

**Effects of NPA and Its Derivatives on Mitochondrial Swelling**

Pi was an essential factor for calcium-induced mPT, and the addition of Ca2+ to mitochondrial suspensions decreased absorbance at 540 nm (an index of mitochondrial swelling) (Figs. 2A–C). In the absence of Pi, the addition of Ca2+ did not significantly affect absorbance (dashed line in Fig. 2A). CsA (2.5 µM) completely inhibited Ca2+-induced changes in absorbance (dotted line in Fig. 2A). MEF (500 µM) induced mitochondrial swelling (solid line in Fig. 2B), which was suppressed by CsA (2.5 µM) as well as the calcium stimulation (dotted line in Fig. 2B). The NSAIDs being tested, except for DPA, had similar mitochondrial swelling profiles (e.g., requirement of Pi and CsA sensitivity) to that of MEF (data not shown). DPA (500 µM) induced Pi-independent mitochondrial swelling (dashed line in Fig. 2C) that was not suppressed by the addition of CsA (dotted line in Fig. 2C).

We examined the dose responses of mitochondrial swelling after 10 min of addition of NPA scaffold NSAIDs. In the presence of Pi, absorbance at 540 nm gradually decreased with
the addition of up to 100 µM MEF, and a marked decrease was observed at 200 µM MEF (Fig. 3B). In the swelling profile of MCL, significant dose-dependent effects were observed up to 100 µM (Fig. 3C). In the swelling profile of TOL, weak effects were noted at 20 µM, whereas a marked change in absorbance was observed at 50 µM (Fig. 3D). The swelling profile of FLU was similar to that of TOL, with a significant decrease in absorbance being noted at 100 µM FLU (Fig. 3E). Weak dose-dependent mitochondrial swelling was detected up to 200 µM of DIC or NPA (Figs. 3F, G). Changes in absorbance were not observed up to 100 µM DPA, while a weak change was noted at 200 µM (Fig. 3H). Mitochondrial swelling induced by the NSAIDs being tested (500 µM), except for DPA, was inhibited by the addition of CsA (2.5 µM) (dotted line in Fig. 2C). DPA (500 µM) induced decreases in absorbance in the absence of Pi (dashed line in Fig. 2C), which suggested that it used a Pi-independent mPT-inducing system.

Uncoupling Effects of NSAIDs on Mitochondrial Oxygen Consumption
Since mitochondrial de-energization (e.g., uncoupling) has been shown to promote the induction of mPT, we examined the effects of NPA scaffold NSAIDs on mitochondrial oxygen consumption (an index of mitochondrial function). In order to inhibit the acceleration of oxygen consumption by mPT, mitochondria pretreated with CsA (2.5 µM) were used, and the states of uncoupling were examined in detail. The basal oxygen consumption rate (state 4) was 25.8 ± 1.6 natomsO/mg/min (solid line in Fig. 4A), and it increased to 116.1 ± 4.8 natomsO/mg/min with the addition of SF6847 (100 nM, positive control of uncoupling) (dotted line in Fig. 4A). MEF (200 µM) decreased the oxygen consumption rate to 52.8% that observed with the addition of SF6847 (dashed line in Fig. 4A). The oxygen consumption rate noted with the addition of 500 µM MEF was similar to that of state 4. The stimulatory effects of MCL, TOL and FLU on oxygen consumption disappeared in a dose-dependent manner (Fig. 4B), and the oxygen consumption rate observed with the addition of 500 µM of these NSAIDs were similar to that of state 4 or less (closed columns in Fig. 4B). These compounds appeared to dose-dependently inhibit the mitochondrial electron transport system. DIC and NPA (200 µM) induced the same oxygen consumption rate as that of MEF (200 µM). An increase in oxygen consumption was not observed with the addition of DPA (Fig. 4B).

Effects of NSAIDs on Mitochondrial Electron Transport
When SF6847 (100 nM)-treated mitochondria (i.e., completely uncoupled mitochondria) were incubated in +Pi medium with a respiratory substrate, rapid oxygen consumption was observed (solid line in Fig. 5A). The effects of the tested compound on electron transport system were examined using completely uncoupled mitochondria. MEF dose-dependently suppressed SF6847-treated mitochondrial oxygen consumption (50, 200, and 500 µM in Fig. 5A). The other NSAIDs tested...
also inhibited SF6847-treated mitochondrial respiratory activity in a dose-dependent manner (Fig. 5B). The control oxygen consumption rate was reduced by 60.4, 66.5, 79.6, 70.1, 47.7, 31.9, and 39.0% following the addition of 200 µM of MEF, MCL, TOL, FLU, DIC, NPA, and DPA, respectively. Thus, these NPA scaffold NSAIDs affected mitochondrial respiration by inhibiting electron transport.

**Molecular Features of Tested NSAIDs**

A conformational analysis (i.e., global minimum search) was performed for the NSAIDs being tested using CONFLEX as previously reported.\(^{21,22}\) MEF had 163 conformers, the energies of which were \(-6.39 \sim -1.89\) kcal/mol (Fig. 6A). Various conformations and energies were obtained for other NSAIDs; MCL (Fig. 6C: 75 conformers, \(-6.39 \sim -10.11\) kcal/mol), TOL (Fig. 6E: 114 conformers, \(-7.64 \sim -0.12\) kcal/mol), FLU (Fig. 6G: 108 conformers, \(-4.35 \sim -0.77\) kcal/mol), DIC (Fig. 6I: 65 conformers, \(1.86 \sim 8.88\) kcal/mol), NPA (Fig. 6K: 24 conformers, \(-9.75 \sim -4.39\) kcal/mol), and DPA (Fig. 6M: 28 conformers, \(-3.875 \sim -0.538\) kcal/mol). An energy jump point was observed in the DIC analysis (arrowhead in Fig. 6I), which indicated that the DIC molecule had a critical point for conformational changes.

The solvation free energies (dGWs) of NSAIDs, which is one of the parameters for stereo-hydrophobicity, were obtained using molecular orbital calculation as previously described (a lower dGW value indicates higher hydrophobicity).\(^{23-25}\) The dGW profile of MEF (\(-66.84 \sim -47.66\) kcal/mol) was shown in Fig. 6B, and displayed large variation in the dGW values obtained. In the dGW analysis of MCL (\(-66.27 \sim -54.68\) kcal/mol), FLU (\(-78.06 \sim -58.35\) kcal/mol) and NPA (\(-70.06 \sim -53.29\) kcal/mol), a minimum dGW group (higher hydrophobic conformers) was obtained (dotted circles...
DISCUSSION

Hepatotoxicity is a known side effect of many kinds of NSAIDs. In the present study, we verified the effects of NPA scaffold NSAIDs on rat liver mitochondrial function as the first step to elucidating the risk of hepatotoxicity associated with these compounds. Morphological changes were induced in mitochondria through a calcium stimulation, and the permeability of the outer and inner membranes significantly increased, resulting in rapidly swelling1-4) (Fig. 2). Significant changes were observed in absorbance (at 540 nm) due to the mitochondrial swelling caused by addition of MEF (200 µM), MCL (100 µM), TOL (50 µM), and FLU (100 µM) (Figs. 3B–E). Weak swelling was induced by the addition of DIC (200 µM), NPA (200 µM), and DPA (200 µM) (Figs. 3F–H). The swelling induced by the NSAIDs being tested, except for DPA, was suppressed by CsA (dotted lines in Fig. 2B versus C). These results indicated that the effects of DPA on the mitochondrial membrane differed from those of the other NSAIDs. Moreover, the existence of Pi was indispensable to mPT by NSAIDs, except for DPA (dashed line in Fig. 2C); therefore, DPA may induce mPT using a distinctive mechanism from those of the other NSAIDs.

The permeability of the mitochondrial inner membrane is a
very important factor for ionic transport regulation and energy metabolism. The energy (ATP) synthesis system that synchronizes with the intermembrane transfer of ions does not function when the mitochondrial inner membrane becomes too permeable, and this has been suggested to occur in an attempt to prevent hepatotoxicity. The uncoupling activity of MEF (200 µM) was 52.8% that of SF6847 (100 nm) (Fig. 4B), while those of MCL, TOL, and FLU at 50 µM were similar to that of MEF at 200 µM, and decreased in a dose-dependent manner (50~500 µM). The movement of protons has been suggested to be controlled in order to obstruct the changes induced in the mitochondrial electron transport system by these NSAIDs, and thus, uncoupling did not occur. The NSAIDs being tested had dual effects on mitochondrial function: uncoupling activity (Fig. 4) and the suppression of electron transport (Fig. 5).

Mitochondrial function was impaired by DPA-induced mitochondrial swelling, and DPA did not exert uncoupling effects on the mitochondrial membrane. The effects of the NSAIDs being tested on mitochondrial functions, which ultimately lead to hepatotoxicity, appeared to differ.

Hydrophobicity is one of the factors regulating reactivity, and stereo-structures have been shown to affect molecular hydrophobicity. SF6847 has 127 conformers, and their dGW values were constant in the vicinity of −63 (average: −62.92 kJ/mol (Fig. 6P)). Each conformer may exhibit interactive hydrophobicity with the mitochondrial membrane and exert uncoupling effects at a very low concentration (100 nm). An individual NSAID molecule has various conformations, and conformers with a particular stereo-structure interact with biological membranes. Various conformers were observed in the conformational analysis of MEF, and large variations were noted in dGW values (Fig. 6B). The ratio of MEF conformers that interacted with the mitochondrial inner membrane appeared to be low, and the dose–response gradually increased until 100 µM MEF (Fig. 3B). The interactive MEF conformer ratio may have been markedly increased by the addition of 200 µM, and the mitochondrial swelling was observed. MCL had a minimum dGW conformer group, and the average (−65.95 kJ/mol, dotted circle area in Fig. 6D) of this group was similar to the dGW level of SF6847. The stereo-hydrophobicity of MCL appears to be advantageous for interactions with biological membranes (e.g. liver mitochondrial membranes), and led to dose-dependent mitochondrial swelling (Fig. 3C). The dGW values of TOL varied (Fig. 6F), and the existence ratio of conformers with appropriate hydrophobicity to interact with mitochondrial inner membranes was low. A sudden swelling was caused by the addition of 50 µM TOL in the dose–response analysis (Fig. 3D). The dGW values of DIC also varied, and there were few advantageous conformers for interactions with inner membranes (Fig. 6J). The swelling reaction was weak following the addition of 200 µM DIC (Fig. 3F). FLU had three dGW groups (gray circle areas in Fig. 6H), which were classified into higher (average: −76.99 kJ/mol), middle (average: −68.34 kJ/mol), and lower (average: −59.28 kJ/mol) hydrophobicity groups. These three groups shifted from the average dGW value of SF6847 (−62.92 kJ/mol), and few interactive conformers with appropriate hydrophobicity existed. Therefore, mitochondrial swelling was not observed until the addition of 20 µM FLU (Fig. 3E), while weak swelling was detected at 50 µM FLU. The minimum dGW group of NPA was constructed with only 8 conformers, its average (−69.43 kJ/mol, dotted circle area in Fig. 6L) differed from the SF6847 average, and a slow dose–response was observed (Fig. 3G). The dGW values of DPA were almost unchanged (average: −24.63 kJ/mol, Fig. 6N) and significantly differed from other NSAIDs, which appeared to be disadvantageous for interactions with biomembranes. The ability of DPA to induce mitochondrial swelling was weak, and was hardly induced by the addition of 100 µM DPA (Fig. 3H). The induction of mitochondrial swelling by DPA was not suppressed by CsA (Fig. 2C), which indicated that DPA had distinctive features from the other NSAIDs being tested. It is reported that the changes in the mitochondrial membrane were caused by DPA-induced hepatotoxicity,11–16 and detailed analysis is being conducted.

The existence probability of conformers with interactive hydrophobicity with biological membranes (e.g. mitochondrial membranes) appears to be one of the key elements for NSAID-induced side effect (e.g. hepatotoxicity), and a detailed analysis is scheduled for the future.

Acknowledgment This work was supported, in part, by a Grant-in-Aid for Scientific Research (15K08113) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of Interest The authors declare no conflict of interest.

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