Structural Requirements for the Hepatotoxicity of Nonsteroidal Anti-inflammatory Drugs in Isolated Rat Hepatocytes

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ABSTRACT
Hepatotoxicity is one of the common side effects of nonsteroidal anti-inflammatory drugs (NSAIDs). We investigated the cytotoxicity of 18 acidic NSAIDs (3 salicylic acids, 3 anthranilic acids, 6 arylacetics, 6 arylpropionic acids) to freshly isolated rat hepatocytes as assessed by the NSAID-induced leakage of lactate dehydrogenase (LDH) in order to determine structural requirements for the direct hepatotoxicity of the NSAIDs. Diflunisal (salicylic acids), flufenamic acid, mefenamic acid, tolfenamic acid (anthranilic acids), diclofenac, indomethacin, acemetacin (arylacetic acids) and flurbiprofen (arylpropionic acids) caused significant LDH leakage, indicating that the LDH leakage was likely to be dependent on the structural characteristics of the compounds. In contrast, the LDH leakage from the noncytotoxic NSAID did not, indicating that the LDH-induced decrease in ATP, probably by their uncoupling effects on mitochondrial oxidative phosphorylation, is involved in the hepatotoxicity of the NSAIDs.

NSAIDs are the most frequently prescribed therapeutic agents (Brooks and Day, 1991). The NSAIDs are used for the treatment of rheumatic and arthritic diseases, because they have analgesic, antipyretic and anti-inflammatory actions, which are mediated by inhibition of the biosynthesis of prostaglandins.

Hepatotoxicity is one of the adverse reactions caused by the NSAIDs (Zimmerman, 1990; Koff, 1992; Park et al., 1992; Rabinovitz and Van Thiel, 1992; Boelsterli et al., 1995). It ranges from mild, transient elevations in serum transaminases to pronounced hepatocellular and/or cholestatic injury, which rarely leads to fatal fulminant hepatitis. This has resulted in the withdrawal of some of these drugs (Koff, 1992; Park et al., 1992). Because some form of hepatic dysfunction has been associated with virtually all of the currently available NSAIDs, the hepatotoxicity is considered as a common characteristic of the NSAIDs. There is no clear statement whether this side effect is caused by a “metabolic idiosyncrasy” or “immunological idiosyncrasy.” As to diclofenac, one of the commonly used NSAIDs, a recent clinical evaluation of the cases reported to the US Food and Drug Administration suggested that the patients with hepatotoxicity by the drug had very few sign of “immunological idiosyncrasy” (Banks et al., 1995), whereas it is difficult to determine whether the “metabolic idiosyncrasy” is a common and a major mechanism of the hepatotoxicity of the NSAIDs. On the other hand, in vitro studies with hepatocytes prepared from the experimental animals have directly shown that some NSAIDs are cytotoxic to the hepatocytes at a concentration close to the respective therapeutic ranges (Akesson and Akesson, 1984; Sorensen and Acosta, 1985; Castell and Sorensen, 1988; Jurima-Romet et al., 1994).

The in vitro comparison of NSAID-induced cytotoxicity showed a large difference between compounds. However, structure requirements for the cytotoxicity, probably based on the mechanism, remains unknown at present. Thus, the purpose of this present study is to examine structure-activity relationships in the hepatotoxicity of NSAIDs in order to clarify their mechanism-based processes. The NSAIDs are a heterogeneous group of compounds, often chemically unrelated, but mostly organic acids. Chemically related acidic NSAIDs, that is, carboxylic acid derivatives were used in the present study. They are generally classified by the substituent position of a carboxyl group, and the classification is

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; LDH, lactate dehydrogenase; DMSO, dimethylsulfoxide; P450, cytochrome P450; UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase.
associated with their pharmacological activities (Brooks and Day, 1991; Boelsterli et al., 1995). We used commercially available 18 acidic NSAIDs, which are thus grouped into 3 salicylic acids, 3 anthranilic acids, 6 arylacetic acids, 6 arylpropionic acids (listed in table 1). The cytotoxicity to freshly isolated rat hepatocytes was assessed by LDH leakage from the hepatocyte, a typical marker for a cell injury. The cytotoxicity of the compounds structurally related to some NSAIDs was also examined. Furthermore, we investigated the effects of NSAIDs on hepatocellular ATP contents as a factor to determine the structure-activity relationships.

Materials and Methods

Chemicals. Diclofenac sodium, salicylic acid, acetysalicylic acid, mefenamic acid, indomethacin, ibuprofen, ketoprofen, flurbiprofen, diphenyl, diphenylamine, collagenase (type I), LDH-UV-Test-Wako, piperonyl butoxide were purchased from the Wako Pure Chemical Ind. (Osaka, Japan). Diflunisal, flufenamic acid, tolfenamic acid, sulindac, zomepirac, ibuprofen, acetamin, naproxen sodium, fenoprofen, suprofen, metyrapone, D-(-)-galactosamine, 4-methylumbelliferone and 4-methylumbelliferyl β-d-glucuronide were from the Sigma Chemical Co. (St. Louis, MO). ATP sodium salt was from the Oriental Yeast Co., Ltd. (Tokyo, Japan). SKF-525A was from the Research Biochemicals Inc. (Natrick, MA). All other chemicals and solvents were of analytical grade.

Preparation of isolated rat hepatocytes. Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals (Saitama, Japan). The animals were housed in air-conditioned rooms (25°C) under a 12 hr light-dark cycle for 1 week before use. Food (commercially available pellet, the Oriental Yeast Co., Ltd.) and water were given ad libitum. Isolated hepatocytes were prepared by the collagenase perfusion method of Moldeus et al. (1978) with modifications. The liver was isolated with perfusion of buffer (pH 7.2) and the hepatocytes were finally suspended in the same buffer. Centrifugation (5000 × g, 2 min). The hepatocytes whose UDPGA contents were reduced were prepared from the rats pretreated with D-(-)-galactosamine (400 mg/kg, ip) 20 min before sacrifice of the rats. All the preparations used in this study were greater than 85% viable as judged routinely by the trypan blue exclusion test.

Incubation of hepatocytes with test compounds. The hepatocytes suspended in the above buffer (2 × 10⁶ cells/ml) were preincubated at 37°C for 5 min. The incubation was started by adding each test compound dissolved in methanol or DMSO. Diclofenac, salicylic acid, acetylsalicylic acid, diflunisal, flufenamic acid, sulindac, zomepirac, ibuprofen, naproxen, ketoprofen, fenoprofen, flurbiprofen, suprofen were dissolved in methanol, and mefenamic acid, tolfenamic acid, indomethacin, acetamin, fenubfen, diphenyl and diphenylamine were dissolved in DMSO. Both of the vehicles were added to the suspension to yield a final concentration of 1%. In the inhibition experiments on P450, SKF-525A (10 μM), piperonyl butoxide (200 μM) or metyrapone (2.5 mM) was added. Aliquots of the suspension were removed from the mixture at appropriate periods during the incubation.

Assay of LDH activity. LDH activities in the supernatant obtained by centrifugation (50 × g, 2 min) of the hepatocyte suspension were assayed with LDH-UV-kit Wako as assessed by oxidation of NADH (Wroblewski and La Due, 1955). Cytotoxicity was expressed as percentage of total LDH activity, which was obtained from the cells treated with 0.5% Triton X-100.

Assay of ATP content. ATP contents were assayed by the HPLC method by Jones (1981) with modifications. The hepatocyte suspension (1 ml) was mixed with 0.5 ml of 3 N HClO₄, followed by addition of 0.25 ml of 6 N KOH and 0.5 ml of 1 M Tris-HCl buffer (pH 7.4). After centrifugation (2000 × g, 10 min), the supernatant with filtration was applied to HPLC. The HPLC conditions were: column, Inertsil ODS (GL Sciences, Tokyo, Japan); mobile phase, 100 mM potassium phosphate buffer (pH 6.0); flow rate, 1.0 ml/min; UV-detection, 259 nm.

Assay of UDPGT activity. UDPGT activity toward 4-methylumbelliferyl β-d-glucuronide was assayed according to the method of Lilenblum et al. (1982). The hepatocyte suspension (0.5 ml) was mixed with 1 ml of 0.5 N HClO₄, followed by an excessive substrate was extracted with 4 ml chloroform. After centrifugation (2000 × g, 10 min), an aliquot (1 ml) of the aqueous phase containing the glucuronide was mixed with 2 ml of 1.6 M glycine/NaOH (pH 10.3). Fluorescence was measured at excitation and emission wavelength of 315 and 365 nm, respectively, as calibrated with 4-methylumbelliferyl β-d-glucuronide as a standard.

Results

Cytotoxicity of diclofenac to isolated rat hepatocytes. Addition of increasing concentrations of diclofenac to rat hepatocytes resulted in decrease in cell viability as assessed by LDH leakage to the incubation medium (fig. 1A). The LDH-leakage was dose and time dependent as was reported by Schmitz et al. (1992). The significant increase in the LDH leakage was observed at the diclofenac concentration and the incubation time of not less than 250 μM and 120 min, respectively. Thus the cytotoxicity of NSAIDs were further compared at the drug concentration of 500 μM and the incubation time of 180 min. Diclofenac caused a rapid decrease in ATP level in hepatocytes, which preceded the LDH leakage, whereas some effect was also observed in the incubation without diclofenac (fig. 1B). The biochemical events observed here were consistent with previous observations with cultured rat hepatocytes (Ponsoda et al., 1995), in which the decrease in the ATP content was relatively gradual.

Comparison of cytotoxicity of 18 NSAIDs in isolated rat hepatocytes. As shown in figure 1, incubation of hepa-
tocytes without NSAID resulted in considerable LDH leakage 180 min after the onset of the incubation. It was also observed in vehicle controls, methanol and DMSO, but was not different from that without either vehicle (fig. 2). The following statistical comparison of the mean value of LDH leakage after incubation with respective NSAIDs was performed with those of corresponding vehicles. In salicylic acid derivatives, only the incubation with diflunisal resulted in LDH leakage as compared with the vehicle under the present conditions. All of the anthranilic acid derivatives used, flufenamic acid, mefenamic acid and tolfenamic acid induced LDH leakage. In arylacetic acid derivatives, diclofenac, indomethacin and acemetacin induced it. In arylpropionic acid derivatives, only the flurbiprofen resulted in LDH leakage as compared with the vehicle. Fenoprofen had a tendency to cause LDH leakage, but was not significant, while significant leakage was observed at the concentration of 1 mM (data not shown).

Effects of NSAIDs on ATP content of isolated rat hepatocytes. Because the reduction of ATP preceded the LDH leakage and it was depleted almost completely 120 min after onset of the incubations (fig. 1), the effects of 18 NSAIDs on the intracellular ATP were compared under the same conditions as the LDH assay except sampling 120 min after onset of the incubation. As shown in figure 3, in addition to diclofenac, all of the NSAIDs leaking LDH (diflunisal, flufenamic acid, mefenamic acid, tolfenamic acid, indomethacin, acemetacin and flurbiprofen) depleted the intracellular ATP extensively. Fenoprofen, which had a tendency to leak LDH, also caused a significant decrease in the ATP content. On the other hand, none of the NSAIDs which did not cause the LDH leakage affected ATP content of the hepatocyte, while fenbufen had a tendency to decrease the intracellular ATP, but was not significant.
Cytotoxicity of the compounds structurally related to the cytotoxic NSAIDs. We tested effects of the compounds structurally related to the cytotoxic NSAIDs, whose substituent groups were completely removed, on LDH leakage in isolated rat hepatocytes in order to investigate the structural requirement for the cytotoxicity of NSAIDs. The “skeletal” compounds and the corresponding NSAIDs were: diphenyl to diflunisal and flurbiprofen; diphenylamine to diclofenac and anthranilic acids. The compounds used here were listed in figure 4. All of the compounds used could be dissolved with DMSO better than with other solvents, but the concentration of diphenyl was limited to 100 μM because of its poor solubility. Thus, the incubation of hepatocytes with diphenyl along with diflunisal was done at the concentration of 100 μM, whereas those with diphenylamine and diclofenac were at the concentrations of 100 μM and 500 μM, respectively. As shown in figure 5, marked LDH leakage was not observed after the incubation with diphenyl or diflunisal at the concentration of 100 μM, while that with diflunisal had a tendency to cause LDH leakage at this concentration.

Incubation with diphenylamine as well as diclofenac resulted in the LDH leakage at 500 μM, but not at 100 μM.

**Effects of diclofenac and diphenylamine on ATP contents of isolated rat hepatocytes.** ATP contents of the isolated rat hepatocytes were determined 120 min after the onset of the incubation with diclofenac or diphenylamine. Diclofenac at 100 μM did not affect the ATP content, whereas diclofenac decreased it slightly but significantly at the concentration of 500 μM (fig. 6).

**Effect of inhibition of drug-metabolizing enzyme activities on diclofenac cytotoxicity.** Diclofenac-induced LDH leakage was determined in the presence or absence of nonselective inhibitors of P450. Addition of noncytotoxic concentration of SKF-525A, metyrapone or piperonyl butoxide did not affect the diclofenac-induced LDH leakage (fig. 7). Hepatocytes with reduced UDPGA contents were prepared.
by *in vivo* pretreatment of rats with D(+)-galactosamine 20 min before preparation of the hepatocytes. The hepatocytes have significantly lower UDPGT activity toward 4-methyl-umbelliferone than those from nontreated rats (data not shown). However, diclofenac induced LDH-leakage in the cofactor-reduced hepatocytes to the same extent as in those of nontreated hepatocytes (fig. 7).

**Discussion**

Previous *in vitro* studies with cultured rat hepatocytes have directly shown that some NSAIDs such as diclofenac are cytotoxic to hepatocytes (Akesson and Akesson, 1984; Sorensen and Acosta, 1985; Castell et al., 1988; Schmitz et al., 1992; Jurima-Romet et al., 1995). *In vitro* study on the NSAID-induced cytotoxicity revealed that there was a large difference in toxic activities between NSAIDs. Furthermore, some drugs were cytotoxic at the concentration close to but not over the respective therapeutic concentrations. Jurima-Romet et al. (1994) compared concentration-dependency in cytotoxicity of 12 NSAIDs to cultured rat hepatocytes. They found diflunisal as the most cytotoxic NSAID, which induced LDH leakage at a concentration not lower than 100 μM. On the other hand, all of the NSAIDs tested were cytotoxic at mM concentrations, indicating that nonspecific cytotoxicity of NSAIDs independent of their chemical structure might be detected at these concentrations. These implies that the NSAIDs at the concentration order of hundred μM is appropriate to elucidate structural and biological determinants giving difference in the cytotoxicity between the NSAIDs, although the concentration range is higher than therapeutic plasma concentrations of the almost all NSAIDs used here.

We investigated the cytotoxicity of 18 acidic NSAIDs (3 salicylic acids, 3 anthranilic acids, 6 ary lacetic acids, 6 arylpropionic acids) to freshly isolated rat hepatocytes as assessed by the NSAID-induced LDH leakage to determine structural requirements for the direct hepatotoxicity of the NSAIDs. Under the condition as decided above, approx. half of the test compounds revealed significant cytotoxicity, that is, diflunisal (salicylic acids), flufenamic acid, mefenamic acid, tolifenamic acid (anthranilic acids), diclofenac, indomethacin, acetamethazin (ary lacetic acids) and flurbiprofen (arylpropionic acids) resulted in significant LDH leakage (fig. 2). That is, one or more cytotoxic NSAIDs are included in the respective group classified by the substituent position of a carboxyl group. This suggests that the structural requirement for the hepatotoxicity of the NSAIDs is not determined by the classification.

The cytotoxic NSAIDs were of three types as classified by their skeleton, that is, diphenyl (diflunisal, flurbiprofen), diphenylamine (diclofenac, flufenamic acid, mefenamic acid, tolifenamic acid) and indolacetic acid (indomethacin, acetamethazin). Among them, indolacetic acid might not be an essential structure, because sulindac was not cytotoxic. Thus, we tested the cytotoxicity of diphenyl and diphenylamine along with diflunisal and diclofenac (fig. 4) to elucidate their contribution to the hepatotoxicity of the corresponding NSAIDs. Incubation of hepatocytes with diphenyl did not cause LDH leakage at the concentration (100 μM) that diflunisal, which has a diphenyl structure, caused it (fig. 5). Nakagawa et al. (1993) compared the cytotoxicity of diphenyl and hydroxydiphenyls in isolated rat hepatocytes, and found that diphenyl itself was not cytotoxic, but potent cytotoxicity was given by the substitution of the hydroxy group(s) into the specific positions. Thus, it is suggested that the substitution of halogen in addition to carboxyl group is important in the cytotoxicity of NSAIDs whose structure is similar to diphenyl. On the other hand, incubation of hepatocytes with diphenylamine induced LDH leakage to the same degree as with diclofenac, which has a diphenylamine structure (fig. 5). Therefore, diphenylamine itself was suggested to contribute to the cytotoxicity of diclofenac and anthranilic acids such as flufenamic acid, mefenamic acid and tolifenamic acid.

Incubation of the hepatocytes with a cytotoxic concentration of diclofenac resulted in rapid decrease in the ATP content, which preceded the LDH leakage (fig. 1). All of the cytotoxic NSAIDs and diphenylamine extensively decreased hepatocellular ATP content, whereas the noncytotoxic NSAID did not (fig. 3). This indicates that the NSAID-induced decrease in ATP is mainly responsible for the cytotoxicity at least under the conditions used in the present study. On the other hand, the incubation with fenoprofen resulted in a marked decrease in the ATP content, although it did not cause a significant LDH leakage. It indicated that the alteration of ATP not only precedes the cytotoxicity but is a sensitive biological response. In addition, it seems that the ATP content has a threshold to directly reduce the viability of the hepatocytes.

A previous study has indicated that some NSAIDs such as diflunisal, mefenamic acid and flufenamic acid have uncoupling effects on oxidative phosphorylation in isolated rat mitochondria (McDougall et al., 1983). The uncoupling oxidative phosphorylation directly could result in inhibition of mitochondrial ATP synthesis. It was also reported that salicylic acid and acetylsalicylic acid, which did not diminish hepatocellular ATP under the present conditions, also had uncoupling effects, but the effects were observed only at higher concentration than the other NSAIDs described above. Recently it has been demonstrated that diclofenac has an uncoupling effect on mitochondrial oxidative phosphorylation, whose potency is close to those of diflunisal and mefenamic acid (Mahmud et al., 1996; Mingatto et al., 1996; Petrescu and Tarba, 1997). These findings imply that the difference between the NSAIDs in the potency as the uncoupler directly reflects on that as the depletor of ATP, whereas drug concentration in mitochondria as well as the intrinsic uncoupling potency is thought to be an important factor to determine the effects on the hepatocellular ATP content.

Nieminen et al. (1994) demonstrated that hepatocyte killing by carbonyl cyanide m-chlorophenylhydrazone, a potent uncoupler of mitochondrial oxidative phosphorylation, was associated with ATP depletion, supporting that inhibition of cellular ATP formation is a crucial event in the progression of irreversible cell injury. Therefore, the ATP depletion induced by the specific NSAIDs, probably attributed to their uncoupling effects on oxidative phosphorylation, is suggested to be closely associated with the cytotoxicity of the NSAIDs, both of which were simultaneously observed here.

Diphenylamine caused a decrease in ATP content to the same degree as diclofenac, suggesting that diphenylamine also has an uncoupling effect, whereas it has not been known as an uncoupler. It has been widely accepted that the uncoupling effect is a common nature of acidic NSAIDs having a
carboxyl group, whose potency varies among NSAIDs as described above (McDougall et al., 1983; Mahmud et al., 1996; Mingatto et al., 1996; Petrescu and Tarba, 1997). However, if diphenylamine, which is not an NSAID, acts as an uncoupler, diclofenac and anthranilic acids trigger the reported uncoupling effects not as the one of the action of the NSAIDs but as of the diphenylamine derivative.

Previous studies with cultured hepatocytes suggested that diclofenac hepatotoxicity depends on its metabolism, that is, a reactive metabolite and/or intermediate was responsible for the cytotoxicity (Jurima-Romet et al., 1994; Ponsoda et al., 1995). Acyl glucuronide was known as a reactive metabolite of diclofenac (Hargus et al., 1994; Kretz-Rommel and Boelsterli, 1994), whereas Kretz-Rommel and Boelsterli (1993) proposed oxidative metabolism rather than glucuronidation was involved in the cytotoxicity of diclofenac. We have expected that the involvement of the metabolic activation clearly appears in freshly isolated hepatocytes, because drug-metabolizing enzyme activities, particularly the monoxygenase activities, were known to markedly decrease with duration of the hepatocyte culture (Paine, 1990; Skett, 1994). However, it should be noted that no effects of three inhibitors of P450 and a depletor of UDPGA were observed on the diclofenac-induced LDH leakage (fig. 7). This indicates that the cytotoxicity of diclofenac was caused by the drug itself but not by its reactive metabolite, although the possibility remains that the concentrations of the inhibitors were insufficient to inhibit diclofenac metabolism, because they were limited due to their own cytotoxicity. These findings and the proposed mechanism that the NSAIDs trigger as the uncoupler of mitochondrial oxidative phosphorylation and reduce cellular ATP contents indicate that the hepatotoxicity of the NSAIDs is “structure dependent” rather than “metabolism dependent.” The discrepancy as to the involvement of a reactive metabolite might partially come from the experimental condition. The duration of the hepatocyte culture may permit the formation of the reactive metabolites enough to contribute to the cytotoxicity even if the drug-metabolizing enzyme activities decreases with the culture time. In contrast, it seems that depletion of cellular ATP observed in the present study is an early event responsible for cytotoxicity of NSAIDs.

In summary, the cytotoxicity induced by acidic NSAIDs in isolated rat hepatocytes is related to the their “skeleton” structure rather than the substituent position of carboxyl group. The diphenylamine was shown to be one of the essential structures in the NSAID-induced hepatotoxicity. The cytotoxic NSAIDs and diphenylamine extensively decreased hepatocellular ATP content, indicating that the decrease, probably by an uncoupling effect on mitochondrial oxidative phosphorylation, is responsible for the hepatotoxicity of the NSAIDs.

References


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