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Direct cytotoxicity of non-steroidal anti-inflammatory drugs in acidic media: model study on human erythrocytes with DIDS-inhibited anion exchanger

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Non-steroidal anti-inflammatory drugs (NSAID) elicit gastric damage through inhibition of the synthesis of prostaglandins that protect gastric cells and direct effect on mucous layer. As the latter effect is not well understood, we used acid hemolysis test in a model study on the cytotoxicity of nine NSAIDs. Human erythrocytes were used as model cells after their band 3 membrane protein was inhibited with DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonate) that strongly suppressed the entry of acid into cytosole and postponed acid-induced hemolysis. These drugs did not produce measurable hemolysis in media buffered at pH 7.2. However, in acidic media (pH 3.4) they markedly reduced to a variable extent the prelytic interval (time spent by acid to accumulate overcritically in cytosole) and time for 50% hemolysis (acid resistance). The cytotoxicity of NSAID to erythrocytes at acidic medium was expressed by the inverse of the concentration ($C_{50\%}$) that reduced twofold acid resistance. It was related to the hydrophobicity of drug as the log of $C_{50\%}$ depended linearly on the log of its critical concentration for the formation of micelles. Hence, the cytotoxicity of NSAIDs to model cells in acidic media apparently involved the transfer of protonated forms and accumulation of the drug and acid into cytosole. We conclude, the protonophore mechanism could be involved in the direct damage of erythrocytes in acidic media. Based on this cytotoxicity the NSAIDs were ranked as aspirin < paracetamol < nimesulide < diclofenac < piroxicam < meloxicam < ibuprofen < naproxen < indomethacin. This is roughly the same row that expresses the relative *in vivo* gastropathogenicity of NSAIDs, hence, it is likely this mechanism might damage gastric epithelial cells by generation of influx of NSAID and back diffusion of acid and producing stress conditions and apoptosis.

1. Introduction

The chief therapeutic effect that NSAIDs deliver to target tissue is based on their ability to inhibit the biosynthesis of prostaglandins [1]. In addition, NSAIDs have been shown to stabilize the structure of biomembranes that contribute to their anti-inflammatory activity [2]. The membrane stabilizing effect was evidenced during model studies, whereby NSAIDs inhibited heat denaturation of albumin and thermal hemolysis of erythrocytes [2].

The stomach secretes acid in high concentration, yet that acid does not damage cells lining the gastric lumen. The property of the stomach responsible for this protection is termed gastric mucosal barrier the formation of which is stipulated by the synthesis of special type of prostaglandins. The main side effect of NSAIDs is their gastric pathogenicity (gastric erosions, bleeding and fall in the gastric transmucosal potential difference). Gastric toxicity of NSAIDs is mediated by inhibition of prostaglandin synthesis. Eventhough NSAIDs stabilize the structure of plasma membrane of cells a direct toxicity to gastric mucosal cells is also evident [3]. The molecular events leading to this direct damage are still unknown. Several effects explaining the direct toxicity of NSAIDs have been pointed out [4] that include back diffusion of acid, generation of oxidative stress, changes in blood flow, activation of neurophils, induction of apoptosis [5] etc.

Alcohol, aspirin, and bile acids can penetrate the mucosal barrier, allowing hydrogenious ions (H^+) to diffuse from the lumen into the mucosal cells in a process called back diffusion. Back diffusion of hydrogen ions through a mucosal barrier that has been altered by aspirin is thought to be involved in gastric pathogenicity [6]. As the nature of the direct damage is not clear the mechanism that generates back diffusion of acid is still not pointed out. In human, after administration of aspirin (600 mg of aspirin in 100 ml of a saline solution), the potential difference falls within 10 min and recovers within 30 to 60 min. Maximum damage appeared within 10 min after aspirin administration. In human, the initial damage, disruption of surface epithelial cells, occurs within moments of aspirin administration whilst intramucosal hemorrhages occur next. Aspirin is a weak acid (pK_a 3.5) the non-ionized form of which is lipid soluble. To cause gastric damage aspirin must penetrate the mucosal cells, and non-ionized, lipid-soluble aspirin is the only form capable of crossing the mucosal membrane [6]. Relatedly, no gastric damage is found when aspirin is administered in a solution buffered to pH 5 that maintains the pH of gastric content sufficiently greater than the pK_a of aspirin.

These data indicate the importance of the transmembrane transfer of NSAIDs for their direct effect on gastric mucosa that is not studied in general. The aim of our study was to elucidate the mechanism thereby a group of NSAIDs directly damage model cells in a medium with pH close to that in stomach. Human erythrocytes with DIDS-inhibited band 3 membrane protein (anion exchanger) were employed as cellular models. The lysis of these cells in low pH-media has been attested to proceed in three steps: transfer of outside acid through the anion exchanger chiefly; generation of oxidative species after the overcritical accumulation of acid within cells and, hence, barrier damage that liberates electrolytes and hemoglobin [7]. The specific inhibition of the anion exchanger with DIDS reduced the transfer rate of acid into cytosole about 30-fold [8] and, relatedly, increased the time interval for retention of hemoglobin by cells in acidic media about 4 times, compared to intact erythrocytes [7]. Thus, in respect to gastric cells that lack specific membrane pathways for the transfer of acid, DIDS-inhibited erythrocytes are relevant models as their permeability to acid is low.

2. Investigations and results

Similarly to the NSAIDs under study uregit is a highly lipophilic substance that contains a carboxylic group dissociable in a pH interval close to that of most NSAIDs.

Table: Model study data on the dependence of direct cytotoxicity of NSAIDs on their hydrophobicity

NSAID	Dosage for oral administration (mg per tablet)	$C_{50\%}$		Critical concentration for the formation of micelles	
		(μg/ml)	(μM)	(μg/ml)	(μM)
Aspirin	500	210	1150	3500	19200
Paracetamol	500	150	990	350	2320
Nimesulide	100	25	100	90	360
Diclofenac	25	15	56	50	187
Piroxicam	20	10	30	30	90
Meloxicam	7,5	5	16	15	48
Ibuprofen	200	3,7	18	12	58
Naproxen	250	2,2	9,5	20	84
Indomethacin	not applied orally	1,2	7,9	25	165
Uregit	not a NSAID	3,2	9,7	7	23

For each drugs the critical concentration for the formation of micelles (inverse to hydrophobicity) and the concentration ($C_{50\%}$) that decreased two times the acid resistance of erythrocytes (inverse to cytotoxicity) are represented

Uregit cannot, however, disrupt the gastric mucosal barrier and produce gastric damage. This explains the use of uregit in this investigation as control substance to NSAIDs. For each agent (NSAIDs plus uregit), the critical concentration for the formation of micelles was determined in 0.9% NaCl-saline at the same pH 3.4 that induced hemolysis (Table). At concentrations below the critical concentration for the formation of micelles, the drugs did not produce measurable hemolysis in 0.9% NaCl-saline at pH 7.2 (data not shown) but strongly accelerated hemolysis at pH 3.4 (Fig. 1). This implies that the presence of NSAID could only cause damage to model cells in acidic media. This result is similar to the finding that bile acids might only cause damage to human red blood cells at pH less than 7.0 [9].

Based on the reduction of light scattering the time dependence of acid hemolysis displayed two phases (Fig. 1, curve 1). During the prelytic phase the light scattering slowly and linearly decreased reflecting the shrinkage of cells caused by the gradual accumulation of acid into the cytosole [7]. At this phase, the bias of the optical density curve was related to the rate at which acid entered cells. Upon reaching the critical acidification of cytosole hemolysis commenced and the light scattering abruptly fell.

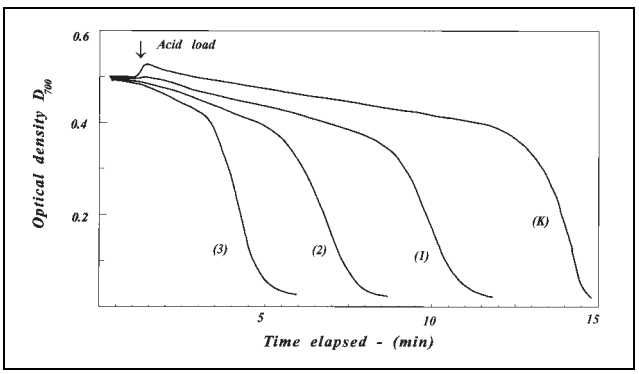

Fig. 1: Impact of various concentrations of NSAID on the time course of HCl-induced hemolysis. Human erythrocytes with anion exchanger inhibited by DIDS were lysed in 0.9% NaCl-saline, pH 3.4, that did not contain (K) or contained diclofenac at concentrations 5,5 mg/ml (1), 11 mg/ml (2) and 22 mg/ml (3). The time for retention of one half of hemoglobin ($T_{50\%}$) is defined as acid resistance of erythrocytes that was linearly reduced by low concentrations of NSAID

Due to the inhibition of the main pathway (band 3 protein) for the penetration of acid the prelytic interval in DIDS-inhibited cells was strongly prolonged compared to the hemolytic phase. Each NSAID shortened the prelytic interval and increased the bias of the optical density curve depending on its concentration in suspension medium (Fig. 1, curves 2, 3 and 4). This demonstrated an increased rate of accumulation of the acid into cytosole through an additional pathway of permeation across the plasma membrane that was different from the band 3 protein.

The increase in the concentration of NSAID present in suspension medium linearly reduced acid resistance until about 40% of its initial value (data not shown). The concentration of NSAIDs that decreased acid resistance two times ($C_{50\%}$) (inverse to the cytotoxicity of NSAID to model cells in acidic media) had lower values with drugs which were more hydrophobic ones (Table). Fig. 2 shows that the log of $C_{50\%}$ concentration was roughly proportional to the log of the critical concentration of the drug for the formation of micelles in 0.9% NaCl-saline at pH 3.4. This indicates that the effect each NSAID produced on acid hemolysis depended on its hydrophobicity and ability to permeate hydrophobic barrier [10].

These findings imply that in acidic media each NSAID played the role of a protonophore to the plasma membranes that was important for the onset of hemolysis. According to Meade et al. [2], when erythrocytes are suspended in neutral media containing NSAID, the drug is incorporated into the plasma membrane and stabilizes its structure, and the more hydrophobic the drug the greater stabilization it produced. When these cells were suspended in acidic (pH 3.4) media with NSAID sufficient number of protonated forms could be found in the media outside cells as the pK_a of each NSAID was near or above that pH. These forms could pass through the hydrophobic barrier of plasma membranes at a rate depending on their lipophilicity, leave protons into the initially neutral cyto-

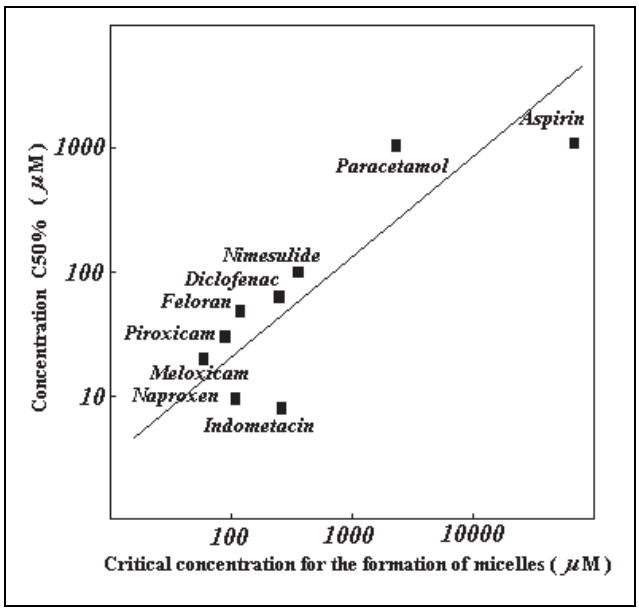

Fig. 2: Direct cytotoxicity of NSAIDs as dependent on their critical concentration for the formation of micelles. The cytotoxicity was expressed by the concentration of NSAID ($C_{50\%}$) that decreased twice the acid resistance of cells. The critical concentration for the formation of micelles was determined as the concentration of NSAID in 0.9% NaCl-saline, pH 3.4, associated with the sharp increase in turbidity

sole and remain there trapped in ionized form. Hence, the more lipophilic a drug, the more pronounced is its direct cytotoxicity to model cells in acidic media (Table). These data indicate that the ability of NSAIDs to enter the cytosole of model cells is important for the cytotoxicity they produce in acidic media.

Previously it has been demonstrated [7] that catalase and superoxide dismutase, which reduce the concentration of hydrogen peroxide and superoxide correspondingly, markedly postponed HCl-induced hemolysis. Now, it was established that the presence of catalase (above 500 U/ml) and superoxide dismutase (above 100 U/ml) in acidic media eliminated the effect of NSAIDs during the hemolysis of model cells and delayed hemolysis (data not shown). These results are in line with the assumed protonophore mechanism through which NSAIDs enter cells and accelerate hemolysis.

3. Discussion

The hemolysis test indicated that NSAIDs exhibited a cytotoxic effect on the model cells (DIDS-inhibited erythrocytes) only in acidic media. The effect apparently depended on the ability of NSAID to augment the influx of acid into cytosole acting as protonophore. In respect to the effect of acid accumulated within the cytosole of model cells, the accumulated NSAID itself apparently produced minor impact on hemolysis. Similar transport process based on the protonophore mechanism could also occur across the plasma membrane of mucosal cells provided the gastric mucosal barrier has been already disrupted. Thus, one might expect the damage produced by NSAIDs on model and mucosal cells to depend on the rate at which the drug and acid accumulate within cytosole. In latter case, however, NSAIDs that accumulated into the cytosole of mucosal cells could be also damaging.

In the Table the NSAIDs under study are ranked according to their cytotoxicity to model cells as expressed by their $C_{50\%}$ concentrations. According to the *in vivo* studies [11] the gastropathogenicities of these NSAIDs are roughly ranked in the same order. Meloxicam is the main exception as it demonstrated marked toxicity to model cells (Table) whilst its *in vivo* pathogenic effect on the mucous layer is mild [11]. This is possibly due to the specific selectivity of meloxicam known to inhibit the synthesis of inflammation-related prostaglandins and, at the same time, preserving the level of protecting prostaglandins in stomach [12]. Thus, meloxicam could not disrupt the gastric mucosal barrier and its permeation in neutral form through the plasma membranes of gastric cells will be low. In line with this explanation, the highly lipophilic ethacrinic acid (uregit) exhibited severe toxicity to model cells (Table), although it is a diuretic not capable to inhibit prostaglandin synthesis and to elicit gastric damage. The rough correlation between the toxicity to model cells and *in vivo* gastropathogenicity of NSAIDs suggests that in case the gastric mucosal barrier has been disturbed the protonophore-related mechanism could be also involved in the direct *in vivo* gastric damage produced by NSAIDs.

It is worthwhile to compare the cytotoxicity of NSAIDs obtained in the model study with their potency to inhibit prostaglandin synthesis. Considering the latter effect, diclofenac has been found more powerful compared to the almost equipotent naproxen, piroxicam and indomethacin, which, in turn, exhibited far greater inhibitory effects than the almost equipotent aspirin and ibuprofen [1]. Thus, the ranking of NSAIDs based on their capacity to damage mucosal cells in stomach [11] and model cells in acidic media (Table) does not correlate with their inhibitory effect on prostaglandin synthesis. This lack of correlation between the gastric injury produced by NSAIDs and their ability to inhibit prostaglandin synthesis has been explained by the more complex mechanism of the cytotoxicity of NSAID that include additional effects on cells in acidic media [4]. The results obtained in this study demonstrate that in case the mucous wall has been disrupted one such effect could be the penetration of NSAIDs into cytosole of mucosal cells using protonophore mechanism. This mechanism allows NSAID to accumulate within the mucosal cells even in the absence of downward concentration gradient using the pH difference that exists between the interior and exterior of cells.

Alongside the accumulation of NSAIDs within mucosal cells, the protonophore mechanism should produce proton influx. Its intensity should be higher with drugs that have greater lipophilicity. The acidification of the interior of mucosal cells should generate free radicals, activate proteolytic enzymes and put a limit on energy consumption that all produce cellular stress. Generally, the cellular stress impairs numerous physiological functions, damages cellular structures, and can lead to cell death. As the NSAIDs stabilize the structure of plasma membranes of cells including the barrier function [2] it is unlikely the cellular death to be of the type of oncosis (initial necrosis). Alternatively, death could occur through the mechanism of apoptosis [13] which is an ordered gene-regulated pathway of self-destruction in response to change in physiological conditions (acidification of cytosole, accumulation of apoptosis-inducing species etc.). Indeed, recent studies have demonstrated that NSAIDs induce apoptosis both in gastric cells and in several lines of tumor cells [14]. Administration of indomethacin to rats (20 mg/kg) caused apoptosis and injury of the gastric mucosal epithelial cells [5]. Cells committing apoptosis were detected at and around the sites of gastric injury. Indomethacin (1–1000 μM) induced apoptosis of primary cultures of gastric epithelial cells in a dose-dependent manner demonstrating direct apoptotic capacity.

During oral administration of various NSAIDs each tablet must contain a specific dose, shown in the Table that produces sufficient benign effect in target tissues and endurable irritation to mucosal cells. Interestingly, for most NSAIDs used in this study, the product of $C_{50\%}$ concentration (Table) and a volume of 2 liters (presumably the average volume of liquid in stomach) was found to be very close to that dose. Thus, the therapeutic concentration of each NSAID in stomach juice is actually very close to the concentration obtained in this study at which the direct toxicity on model cells was apparent. This finding also supports the involvement of the protonophore mechanism in NSAID-related gastric damage. The only exceptions are naproxen and ibuprofen as their therapeutic concentrations in stomach juice are more than 10 times higher than their cytotoxic concentrations obtained during this model study. However, the latter drugs are known for their specific inhibition of the synthesis of inflammatory prostaglandins and the markedly weaker inhibitory effect they produce on the synthesis of protecting prostaglandins [1]. This allows higher dose of these drugs to be administered without compromising the mucous barrier thus preventing the consequent influx of drug and acid into mucosal cells.

In an acid hemolysis test we took the advantage to investigate the direct toxicity that nine NSAIDs produced on erythrocytes as expressed by the decrease in acid re-

sistance. The toxicity of NSAIDs to model cells clearly depended on the ability of each drug to permeate the hydrophobic barrier of plasma membrane in protonated form. For each drug the direct toxicity was found roughly proportional to its *in vivo* gastropathogenicity in support of the conception that this type of toxic activity could be also involved in the direct gastric damage that NSAIDs inflict on gastroepithelial cells. The results presented could contribute to the understanding of NSAID cytotoxicity and help the development of more safety drugs for NSAID therapy.

3. Experimental

The drugs were obtained commercially as pure substances from Sigma (aspirin, indomethacin) or from tablets (paracetamol, nimesulide, diclofenac, piroxicam, meloxicam, ibuprofen, naproxen, and uregit). DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid), catalase (EC.1.11.1.6 from bovine liver) and superoxide dismutase (EC.1.15.1.1 from bovine erythrocytes) were purchased from Sigma Chemical Co, St. Louis, MO, USA. Human erythrocytes from healthy donors were isolated and washed, and their membrane exchanger for anions (band 3 protein) inhibited with DIDS as previously described [7]. For hemolysis test the latter cells were suspended in 0.9% NaCl-saline without (control) or with indicated concentration of NSAID. Each drug was dissolved in hot (50 °C) dimethylsulfoxide (DMSO) and added to the suspension media at a concentration well below its limit of solubility. The hemolysis of cells was induced at room temperature injecting 20 µl diluted (1% v/v) HCl to 1.8 ml continuously stirred suspension (final pH 3.4). The acid hemolysis was followed recording optical density at 700 nm that depended mainly on the light scattering of cells still remaining intact [9]. The time for retention of 50% hemoglobin is arbitrarily defined as a measure for acid resistance of cells.

With the exception of paracetamol (pK_a 9.5) the remaining compounds under study are weak organic acids with pK_a between 3.5 (aspirin) and 6.3 (piroxicam). Relatedly, they are to a variable extent hydrophobic substances and at concentrations above some specific values form micelles in water. Following Albert [10], the concentration that produced a sharp rise in turbidity (700 nm) during the incremental addition of NSAID to 0.9% NaCl-saline was determined and latter referred to as critical concentration of the drug for the formation of micelles. The inverse of this concentration is a measure for the hydrophobicity (lipophilicity) of the protonated form of drug.

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