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Diclofenac-induced stimulation of SMCT1 (SLC5A8) in a heterologous expression system: A RPE specific phenomenon ☆

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ABSTRACT

SMCT1 is a Na^+-coupled monocarboxylate transporter expressed in a variety of tissues including kidney, thyroid, small intestine, colon, brain, and retina. We found recently that several non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of SMCT1. Here we evaluated the effect of diclofenac, also a NSAID, on SMCT1. SMCT1 cDNA was expressed heterologously in the human retinal pigment epithelial cell lines HRPE and ARPE-19, the human mammary epithelial cell line MCF7, and in *Xenopus* laevis oocytes. Transport was monitored by substrate uptake and substrate-induced currents. Na^+-dependent uptake/current was considered as SMCT1 activity. The effect of diclofenac was evaluated for specificity, dose–response, and influence on transport kinetics. To study the specificity of the diclofenac effect, we evaluated the influence of this NSAID on the activity of several other cloned transporters in mammalian cells under identical conditions. In contrast to several NSAIDs that inhibited SMCT1, diclofenac stimulated SMCT1 when expressed in HRPE and ARPE-19 cells. The stimulation was marked, ranging from 2- to 5-fold depending on the concentration of diclofenac. The stimulation was associated with an increase in the maximal velocity of the transport system as well as with an increase in substrate affinity. The observed effect on SMCT1 was selective because the activity of several other cloned transporters, when expressed in HRPE cells and studied under identical conditions, was not affected by diclofenac. Interestingly, the stimulatory effect on SMCT1 observed in HRPE and ARPE-19 cells was not evident in MCF7 cells nor in the *X. laevis* expression system, indicating that SMCT1 was not the direct target for diclofenac. The RPE-specific effect suggests that the target of diclofenac that mediates the stimulatory effect is expressed in RPE cells but not in MCF7 cells or in *X. laevis* oocytes. Since SMCT1 is a concentrative transporter for metabolically important compounds such as pyruvate, lactate, β-hydroxybutyrate, and nicotinate, the stimulation of its activity by diclofenac in RPE cells has biological and clinical significance.

1. Introduction

Recently, we cloned SMCT1, a Na^+-coupled transporter for a variety of monocarboxylates, including lactate, pyruvate, nicotinate, β-hydroxybutyrate, and short-chain fatty acids [1–5]. SMCT1 is expressed in thyroid gland, kidney, small intestine, colon, and brain, and its expression is silenced in cancer [6–9]. The transporter is expressed also in retina with cell-type specific distribution [10]. Since SMCT1 exhibits broad substrate selectivity, its physiologic function may vary from tissue to tissue depending on the relevance of specific substrates to a given tissue. SMCT1 plays an obligatory role in the reabsorption of lactate, pyruvate, nicotinate, and β-hydroxybutyrate in the kidney [11,12]. In the intestinal tract, the transporter may mediate concentrative entry of short-chain fatty acids into absorptive cells. These short-chain fatty acids are generated in the intestinal lumen by bacterial fermentation of dietary fiber. In the brain where the expression is restricted to neuronal cells, it is likely that the transporter mediates concentrative entry of the energy-rich metabolites lactate and β-hydroxybutyrate to maintain energy status of these cells under conditions of reduced supply of glucose [4]. The same may be true in retinal neurons. Since the transporter is also expressed in RPE that constitutes the outer retinal–blood barrier, we believe that it functions in the delivery of lactate, pyruvate, β-hydroxybutyrate, and nicotinate into retina.

Abbreviations: SMCT1, sodium-coupled monocarboxylate transporter 1; NSAIDs, non-steroidal anti-inflammatory drugs; RPE, retinal pigment epithelium.

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We reported recently that several non-steroidal anti-inflammatory drugs (NSAIDs) interact with SMCT1 [13]. The function of the transporter is inhibited by ibuprofen, ketoprofen, fenoprofen and naproxen, with ibuprofen being the most potent inhibitor. Even though ibuprofen is a monocarboxylate, it is not a transportable substrate for SMCT1; instead, it is a blocker of the transporter. These findings may have clinical and pharmacologic relevance to retina. Recent data suggest that NSAIDs may be beneficial in the treatment of ocular diseases/complications affecting the posterior segment, including diabetic retinopathy and macular edema [14–16]. Since SMCT1 in RPE is likely to mediate the delivery of important metabolites/nutrients into retina, NSAIDs may interfere with this important function of RPE. In the present study, we examined the effects of additional NSAIDs on SMCT1. These studies have produced unexpected results. Contrary to what was observed with NSAIDs such as ibuprofen, some NSAIDs such as diclofenac exhibit marked stimulatory effects on SMCT1 expressed heterologously in RPE cell lines. Interestingly, the stimulatory effect was not observed when SMCT1 was expressed heterologously in MCF7 cells or in *Xenopus* laevis oocytes, suggesting that the stimulatory effect of diclofenac is indirect, mediated through some other, hitherto unidentified, protein that is expressed in RPE cells but not in MCF7 cells or *X. laevis* oocytes.

2. Materials and methods

2.1. Materials

[^{14}C]-β-D-Hydroxybutyrate, [^{14}C]-L-lactate, [^{14}C]-(methylamino)isobutyric acid (MeAIB), [^{3}H]methylphenylpyridinium (MPP^+) iodide and [^{14}C]tetraethylammonium (TEA^+) bromide were purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]Nicotinate and [^{3}H]-L-carnitine were from Moravek Biochemicals, Inc. (Brea, CA). [1,2-^{3}H]Taurine was purchased from Amersham Life Sciences (Piscataway, NJ). Diclofenac, sulindac, and meclofenamate were from Sigma (St. Louis, MO). Cell culture reagents were from invitrogen (Carlsbad, CA).

2.2. Animals

Frogs (*Xenopus laevis*) were used for collection of oocytes. The research protocol for the use of frogs in the present study was approved by the Institutional Committee for Animal Use in Research and Education.

2.3. Cells

The human retinal pigment epithelial cell lines HRPE and ARPE-19 were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium; the human mammary epithelial cell line MCF7 was cultured in DMEM. All culture media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.4. Heterologous expression of SMCT1 cDNAs in HRPE, ARPE-19 and MCF7 cells

The human, mouse, and rat SMCT1 cDNAs were expressed in HRPE cells using the vaccinia virus expression technique [1–4]. Additional studies involved heterologous expression of human SMCT1 in ARPE-19 and MCF7 cells. Uptake of substrates for SMCT1 ([^{14}C]nicotinate, [^{14}C]-β-hydroxybutyrate, or [^{14}C]lactate), was measured with a 15 min incubation (linear uptake rates) at 37 °C. Uptake measurements in cells transfected with vector alone were made in parallel. SMCT1-mediated transport was determined by subtracting uptake measured in vector-transfected cells from that measured in cDNA-transfected cells. This was done to correct for endogenous uptake activity. The uptake buffer was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Two other anti-inflammatory agents, namely meclofenamate and sulindac, were also examined for their effects on SMCT1. Kinetic analysis was performed to determine the effects of diclofenac on substrate affinity and maximal velocity of SMCT1. The kinetic parameters, Michaelis constant (K_t) and maximal velocity (V_{max}) were determined according to Michaelis–Menten equation using non-linear and linear regression methods. To assess the specificity of diclofenac effect on SMCT1, additional cloned human transporters (OCTN1, OCTN2, OCT3, ATA1, ATA2, and TauT) were expressed heterologously in HRPE cells, and the effect of diclofenac on these transporters was examined. The activities of OCTN1 (an organic cation transporter) [17], OCTN2 (a Na^+-coupled carnitine transporter) [18], OCT3 (an electrogenic organic cation transporter) [19], ATA1 and ATA2 (Na^+-coupled amino acid transporters) [20,21], and TauT (a Na^+/Cl^--coupled taurine transporter) [22] were monitored with appropriate substrates.

2.5. Functional expression of human SMCT1 in Xenopus laevis oocytes

Human SMCT1 was expressed in *X. laevis* oocytes as described previously [1] and the activity of the transporter was monitored by lactate uptake as well as by lactate-induced currents. Mature oocytes were injected with 50 ng SMCT1 cRNA. Water-injected oocytes served as controls. The oocytes were used for uptake and electrophysiological studies 3–7 days after cRNA injection. Uptake of [^{14}C]lactate (5 μCi/assay; lactate concentration, 10 μM) into water-injected and SMCT1-expressing oocytes was measured at room temperature with a 60-min incubation in a NaCl-containing buffer in the presence and absence of diclofenac (25 μM). Electrophysiological studies were performed by the two-microelectrode voltage-clamp method [1]. Oocytes were superfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes/Tris, pH 7.5) followed by the same buffer containing lactate. The membrane potential was clamped at −50 mV. When the effects of diclofenac and ibuprofen were studied, these compounds were present along with lactate at a concentration of 25 μM.

2.6. Data analysis

Experiments with expression of SMCT1 cDNAs in HRPE, ARPE-19, and MCF7 cells were repeated three times with independent transfections, and transport measurements were made in duplicate in each experiment. Electrophysiological measurements were repeated at least three times with separate oocytes. Uptake measurements in oocytes were made with 8–10 oocytes, determining the uptake in each oocyte independently. Data are presented as means ± SE.

3. Results

3.1. Stimulation of SMCT1 by diclofenac in a mammalian cell expression system

Recently, we reported that certain non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen interfered with the function of human SMCT1 [13]. This effect was observed in two different heterologous expression systems: a mammalian cell expression system and the *X. laevis* oocyte expression system. When we screened additional NSAIDs for their effects on SMCT1 using the

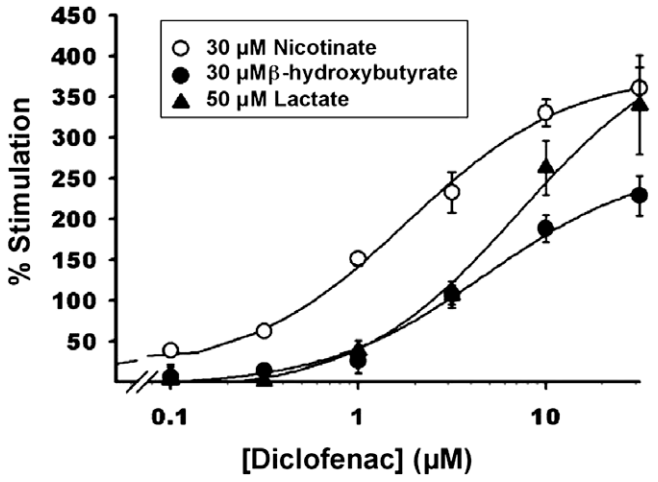

Fig. 1. Dose–response relationship for the diclofenac-induced stimulation of human SMCT1 in HRPE cells. Human SMCT1 cDNA was expressed functionally in HRPE cells by the vaccinia virus expression technique. Transport activity of SMCT1 was monitored by measuring the uptake of three different substrates: nicotinate (30 μM), β-hydroxybutyrate (30 μM) or lactate (50 μM). Uptake was measured for 15 min in the presence of NaCl and in the presence of increasing concentrations of the NSAID diclofenac. Data represent percent stimulation of uptake in the presence of various concentrations of diclofenac compared to control uptake measured in the absence of diclofenac.

mammalian cell expression system, we discovered that diclofenac did not interfere with the function of human SMCT1, but actually had a marked stimulatory effect. This was an unexpected finding. Fig. 1 describes the dose–response relationship for the stimulatory effect of diclofenac on human SMCT1 with three different substrates: nicotinate, lactate, and β-hydroxybutyrate. The maximal stimulation was 2- to 5-fold, and half-maximal stimulation occurred at a diclofenac concentration of 2–5 μM. This effect was not species-specific. Diclofenac stimulated mouse SMCT1 and rat SMCT1 as well (Table 1). But, the observed stimulatory effect was specific for SMCT1; there was no effect of diclofenac on six other transporters when monitored under identical conditions (Table 2). The transporters examined were: human OCTN1 (a novel organic cation transporter which transports tetraethylammonium in a Na^+-independent manner), human OCTN2 (a Na^+-coupled transporter for carnitine), human OCT3 (an electrogenic organic cation transporter which transports methylphenylpyridinium), human ATA1 and human ATA2 (two different amino acid transporters which mediate Na^+-coupled transport of the model amino acid substrate (methylamino)isobutyric acid), and human TauT (a Na^+/Cl^--coupled transporter for taurine).

Table 1
Influence of diclofenac (25 μM) on the transport of β-hydroxybutyrate mediated by SMCT1 from different species.

Transporter	β-Hydroxybutyrate Uptake			
	Control		Diclofenac	
	pmol/10^6 cells/15 min	%	pmol/10^6 cells/15 min	%
Human SMCT1	111.2 ± 10.3	100	583.5 ± 33.7	525
Rat SMCT1	239.4 ± 41.8	100	943.6 ± 50.1	394
Mouse SMCT1	178.7 ± 15.0	100	849.1 ± 31.5	475

SMCT1 cDNAs from different species are expressed heterologously in HRPE cells by the vaccinia virus technique. Cells transfected with vector alone served as the control to adjust for endogenous uptake activity. Uptake of β-hydroxybutyrate (50 μM) was measured for 15 min in the presence of NaCl with and without diclofenac (25 μM). SMCT1-specific uptake was calculated by subtracting uptake measured in vector-transfected cells from uptake measured in cDNA-transfected cells. Data represent means ± SE.

Two other NSAIDs that showed a stimulatory effect on SMCT1 were meclofenamate and sulindac. Fig. 2 describes the dose–response relationship for the stimulation of human SMCT1 by these NSAIDs. The maximal stimulation with meclofenamate was comparable to that observed with diclofenac, but the concentration eliciting half-maximal stimulation was much higher than observed with diclofenac (~3 μM versus ~30 μM).

We then performed additional studies to determine which of the kinetic parameters of SMCT1-mediated transport process was affected by diclofenac (Fig. 3). Both kinetic parameters, namely the Michaelis constant (K_t) and the maximal velocity (V_{max}), were modified by diclofenac. K_t was reduced from 778 ± 121 μM to 299 ± 26 μM, but V_{max} was increased from 1.5 ± 0.2 nmol/10^6 cells/min to 2.3 ± 0.1 nmol/10^6 cells/min, in the presence of diclofenac (25 μM).

3.2. Effect of diclofenac on SMCT1 expressed heterologously in X. laevis oocytes

We then used the *X. laevis* oocyte expression system to confirm the stimulatory effect of diclofenac on human SMCT1. The function of the transporter was monitored by two different methods. We first used lactate uptake as the read-out for SMCT1 activity. The uptake of lactate (10 μM) in the presence of NaCl increased more than 30-fold in cRNA-injected oocytes compared to water-injected oocytes (Fig. 4A). However, diclofenac (25 μM) did not have any significant effect on this transport process. This was unexpected based on the marked stimulation of the transporter in the mammalian cell expression system. Therefore, we employed a different approach to monitor the function of SMCT1. Since SMCT1 is an electrogenic transporter, its transport function can be monitored as substrate-induced inward currents under voltage-clamp conditions. As expected, superfusion of SMCT1-expressing oocytes with lactate (1 mM) induced inward currents (Fig. 4B). Such currents were not detected in water-injected oocytes (data not shown), indicating that the observed currents were a genuine read-out of SMCT1 function. SMCT1-expressing oocytes were superfused with lactate (1 mM) and diclofenac (25 μM) together. There was no change in the magnitude of lactate-induced currents with or without diclofenac. In contrast, the presence of ibuprofen (25 μM) along with lactate (1 mM) reduced lactate-induced currents. These data indicate that diclofenac has no effect on SMCT1 when expressed in *X. laevis* oocytes. The results with ibuprofen are congruent with our earlier findings that ibuprofen is a blocker of SMCT1.

3.3. Cell type specificity of diclofenac effect on SMCT1 in the mammalian cell expression system

The lack of stimulatory effect on SMCT1 by diclofenac in the oocyte expression system came as a surprise because this suggests that the transporter may not be the direct target for diclofenac. If it were, SMCT1 expressed in oocytes should have been stimulated by diclofenac. We hypothesized that HRPE cells, used in the mammalian cell expression system, may express a protein, hitherto unidentified, which mediates the stimulatory effect of diclofenac on SMCT1. To test this hypothesis, we used two additional cell lines, ARPE-19 (a human RPE cell line) and MCF7 (a human mammary epithelial cell line), for heterologous expression of human SMCT1 and examined the effect of diclofenac (Fig. 5). We found diclofenac to be an effective stimulator of SMCT1 in HRPE cells and ARPE-19 cells. However, there was no effect on SMCT1 when expressed in MCF7 cells. This indicates that the protein responsible for the stimulatory effect of diclofenac is expressed in the RPE cell lines but not in the mammary epithelial cell line.

Table 2
Differential influence of diclofenac on SMCT1.

Transporter	Substrate	Transport activity			
		Control		Diclofenac	
		pmol/10^6 cells/15 min	%	pmol/10^6 cells/15 min	%
Human SMCT1	Lactate	726.1 ± 43.8	100	3311.6 ± 66.6	456
Human OCTN1	TEA^+	301.5 ± 37.1	100	365.4 ± 31.6	121
Human OCTN2	Carnitine	6.4 ± 0.5	100	6.5 ± 0.3	102
Human OCT3	MPP^+	464.3 ± 43.8	100	456.6 ± 46.1	98
Human ATA1	MeAIB	235.8 ± 8.1	100	249.6 ± 10.1	106
Human ATA2	MeAIB	2869.8 ± 493.2	100	2312.0 ± 134.2	81
Human TauT	Taurine	21.7 ± 0.8	100	23.3 ± 0.9	107

TEA^+, tetraethylammonium; MPP^+, methylphenylpyridinium; MeAIB, α-(methylamino)isobutyric acid.
Various cloned transporter cDNAs were expressed heterologously in HRPE cells by the vaccinia virus technique. Vector-transfected cells were used as a control to adjust for endogenous uptake activity. Transport function of various transporters was monitored by the uptake of appropriate substrates in the absence or presence of diclofenac (25 μM). Data (means ± SE) represent only cDNA-specific uptake.

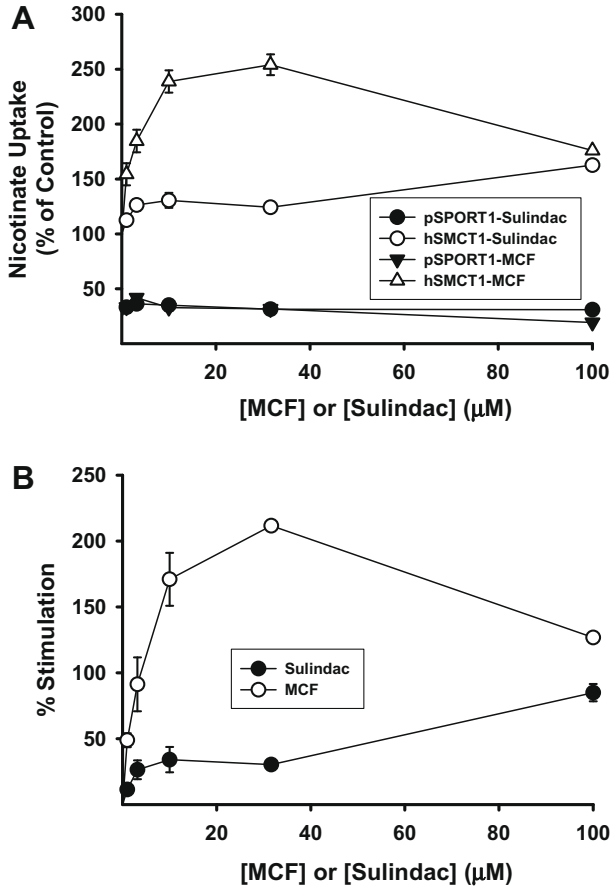

Fig. 2. Dose–response relationship for the stimulation of human SMCT1 by the non-steroidal anti-inflammatory drugs meclofenamate (MCF) and sulindac in HRPE cells. HRPE cells were transfected with either pSPORT1 vector alone or human SMCT1 cDNA. The activity of SMCT1 was monitored in the form of nicotinate uptake. (A) Uptake of [^{14}C]nicotinate (30 μM) was measured in the presence of NaCl and in the presence of increasing concentrations of MCF or sulindac. (B) Data represent percent stimulation of uptake in the presence of various concentrations of MCF or Sulindac compared to control uptake measured in the absence of MCF or Sulindac.

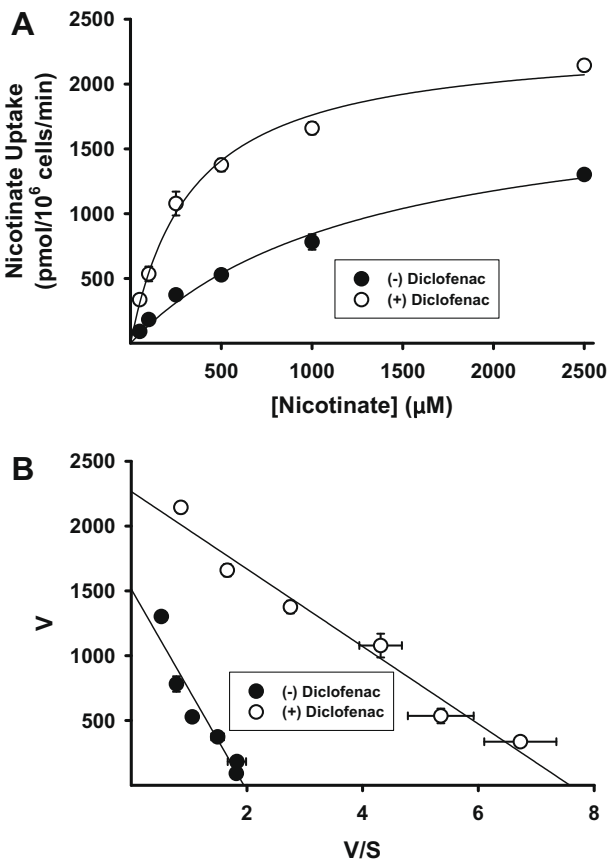

Fig. 3. Influence of diclofenac on the kinetics of human SMCT1-mediated nicotinate uptake in HRPE cells. (A) Uptake of nicotinate was measured for 15 min in the presence of NaCl and in the absence or presence of diclofenac (25 μM) with varying concentrations of nicotinate. The concentration of [^{14}C]nicotinate was kept constant (30 μM). (B) Eadie–Hofstee plot (v/s versus v, where v is nicotinate uptake in pmoles/10^6 cells/15 min and s is nicotinate concentration in μM).

4. Discussion

NSAIDs are one of the most widely used forms of ocular drugs. The efficacy of topically administered NSAIDs for reduction of inflammation and pain in the anterior segment is well established. Recent studies, however, suggest that NSAIDs may also be beneficial in the treatment of inflammatory diseases affecting the posterior segment [14–16]. In a previous study, we screened a variety of pharmacologic agents containing a monocarboxylate group as potential substrates for human SMCT1 [13]. This search yielded very interesting, clinically and therapeutically relevant findings with regard to NSAIDs. With a heterologous expression system using the HRPE cell line, we found that the activity of the cloned human SMCT1 was inhibited by ibuprofen, ketoprofen, fenoprofen and naproxen. Dose–response studies demonstrated that ibuprofen was the most potent inhibitor. Interestingly, ibuprofen was not a transportable substrate for SMCT1 but rather a blocker. In

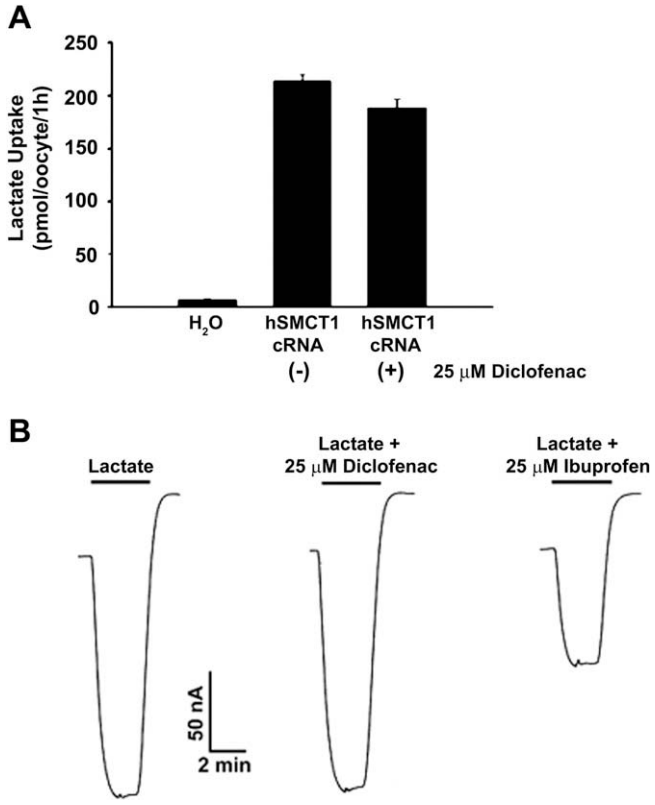

Fig. 4. Differential effects of diclofenac and ibuprofen on human SMCT1 expressed heterologously in *X. laevis* oocytes. (A) Uptake of [^{14}C]lactate (10 μM) was measured in water-injected oocytes and in SMCT1-expressing oocytes in the presence or absence of diclofenac (25 μM). (B) The effects of diclofenac and ibuprofen on lactate-induced currents were analyzed in SMCT1-expressing oocytes. The oocytes were superfused, in the presence of NaCl, with lactate (1 mM) in the absence or presence of 25 μM diclofenac or 25 μM ibuprofen.

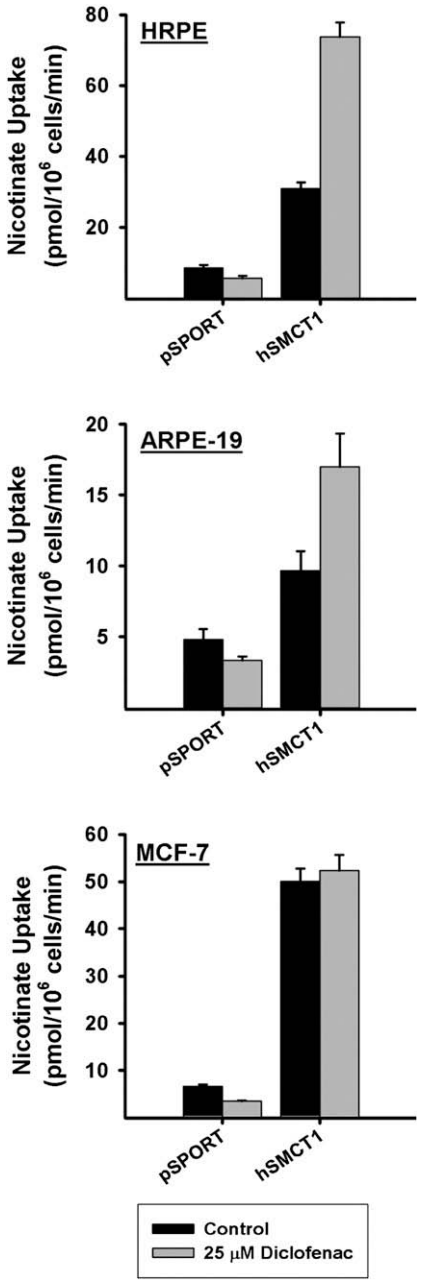

Fig. 5. Cell-type specificity of diclofenac effect on human SMCT1 expressed heterologously in HRPE, ARPE-19 and MCF7 cells. Cells were transfected with either pSPORT1 vector alone or SMCT1 cDNA. Uptake of [^{14}C]nicotinate (30 μM) was measured in the presence of NaCl and in the absence or presence of 25 μM diclofenac.

the present study, we tested the effect of the NSAID diclofenac on SMCT1. In the HRPE cell expression system, we found that diclofenac is a potent activator of human SMCT1. This differs markedly from the effects observed with ibuprofen and other NSAIDs in our previous study [13]. The activation of SMCT1 induced by diclofenac was evident with three different substrates: lactate, β-hydroxybutyrate and nicotinate. The effect of diclofenac was also observed with rat and mouse orthologs of the transporter. The diclofenac-induced activation of SMCT1 is not a non-specific effect, because the activities of several other cloned human transporters, when expressed heterologously in the same cell line using an identical experimental technique, were not affected by diclofenac.

We then proceeded to investigate the effects of diclofenac using the *X. laevis* oocyte expression system, expecting a similar stimulatory effect on SMCT1, but the results were totally unexpected. Diclofenac did not have any effect on SMCT1-mediated lactate uptake in oocytes. The lack of diclofenac effect on SMCT1 in this expression system was confirmed also by using lactate-induced inward currents as the index of SMCT1 function. In our previous study, we found that SMCT1 was a direct target for ibuprofen to produce the blocking effect since the effect was seen in the HRPE cell expression system as well as in the *X. laevis* oocyte expression system [13]. In contrast, the results of our present study demonstrate that diclofenac does not act on SMCT1 directly. If SMCT1 were to be the direct target for diclofenac, the effect should have been seen in both expression systems.

We then used two different mammalian cell lines for heterologous expression of human SMCT1 to determine whether the stimulatory effect of diclofenac is specific for the RPE cell line or whether the phenomenon is common to all mammalian cells. For this purpose, we used MCF7 cells, a human mammary epithelial cell line, and another human retinal pigment epithelial cell line, ARPE-19. As we saw with the HRPE cell line, we were able to detect the stimulatory effect of diclofenac on SMCT1 in ARPE-19 cells, but this phenomenon was not observed in MCF7 cells. These data suggest that the stimulatory effect of diclofenac on SMCT1 is unique to RPE cell lines.

Though the exact mechanisms of the stimulatory effect of diclofenac on Na^+-coupled monocarboxylate transport in RPE cells remains unknown, the finding itself is of clinical significance. The stimulation of Na^+-coupled transport of monocarboxylates in RPE cells by diclofenac was robust; the maximal stimulation was 2- to 5-fold and half-maximal stimulation was observed at a

diclofenac concentration of 2–5 μM. Kinetic analysis of the diclofenac effect on SMCT1 indicated that the stimulation involved an increase in substrate affinity as well as an increase in maximal velocity. The concentration of diclofenac at which significant stimulation was observed is clinically relevant. Plasma levels of diclofenac in humans who take the drug for therapeutic purposes have been reported to be in the range of 0.1–10 μM [23]. Therefore, it is likely that the transport of monocarboxylates in RPE cells via Na^+-coupled processes is stimulated significantly by diclofenac at therapeutic concentrations. This is also true for meclofenamate, another NSAID that stimulates SMCT1 transport activity. Half-maximal effect was observed in the present study at ~30 μM. The plasma levels of this drug at therapeutic doses in humans are in the range of 15–50 μM [20]. The substrates for SMCT1 include lactate, pyruvate, β-hydroxybutyrate, and nicotinate. Lactate and β-hydroxybutyrate are important energy substrates for neurons in the brain and in retina, and nicotinate is an important B-complex vitamin. Pyruvate is an endogenous inhibitor of histone deacetylases that regulate gene expression [24,25]. Thus, all four monocarboxylates are essential for normal retinal health, and SMCT1-mediated transfer of these monocarboxylates across the RPE cell layer is a key determinant of the availability of these essential metabolites/nutrients to the retina. As such, the present findings that diclofenac and meclofenamate may potentially stimulate this process in vivo at therapeutic concentrations of these drugs have significant clinical implications.

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