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Methionine Sulfoxide Reductase A in Human and Mouse Tissues is Responsible for Sulindac Activation, Making a Larger Contribution than the Gut Microbiota[S]

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ABSTRACT

Sulindac is a nonsteroidal anti-inflammatory prodrug that is converted to its pharmacologically active metabolite, sulindac sulfide, via a reduction reaction. It is widely accepted that the gut microbiota is responsible for sulindac activation; however, sulindac-induced gastrointestinal injury, which is caused by irritation of the gastrointestinal tract by its active metabolite, is uncommon. Therefore, it is surmised that sulindac is converted to its active metabolite in tissues after absorption. In this study, we sought to identify the enzyme(s) responsible for sulindac activation in tissues and to compare its/their contribution to the gut microbiota. Sulindac is enzymatically reduced in human intestinal, liver, and renal cytosols. Since sulindac is known to be reduced by methionine sulfoxide reductase (Msr) in *Escherichia coli*, we investigated whether the human ortholog MSRA catalyzes the sulindac reduction reaction. We found that recombinant human MSRA shows sulindac reductase activity with a similar Michaelis constant value as tissue cytosols. In addition, it was revealed that cytosolic factor(s) efficiently enhanced MSRA activity. By using the relative expression factor, the contribution of MSRA to the sulindac reductase activities in each tissue cytosol was calculated to be almost 100%. In mice, depletion of the gut microbiota by administration of antibiotics resulted in a 31% decrease in the area under the curve ratio of sulindac sulfide to sulindac, indicating that the contribution of tissue MsrA to sulindac activation is expected to be 69% in the body. In conclusion, we demonstrated that MSRA expressed in tissues is involved in sulindac activation, making a larger contribution than the gut microbiota.

SIGNIFICANCE STATEMENT

Methionine sulfoxide reductase A is responsible for the activation of sulindac, a nonsteroidal anti-inflammatory prodrug, to sulindac sulfide, an active form, in human tissues. Methionine sulfoxide reductase A expressed in tissues activates sulindac with a higher contribution than gut microbiota in body.

Introduction

Prodrugs are compounds converted to pharmacologically active metabolites after administration. Many prodrugs have been developed with the aim of reducing side effects or improving absorption, stability, water solubility, duration of action, and tumor selectivity (Abet et al., 2017). Most prodrugs are metabolized to active forms by hydrolases, such as carboxylesterases and arylacetamide deacetylase, whose substrate specificities have been gradually uncovered (Fukami et al., 2015; Cerny, 2016). In addition, some prodrugs, such as loxoprofen and oxcarbazepine, are activated by reductases such as aldo-keto reductase (AKR) and short-chain dehydrogenase/reductase families (Tanaka, 1993; Lloyd et al., 1994). The gut microbiota also participates in the activation of prodrugs such as prontosil and salazosulfapyridine (Gingell et al., 1971; Klotz, 1985) by catalyzing reduction reactions. Studies on reductases are lagging behind those on other drug-metabolizing enzymes, such as cytochrome P450, UDP-glucuronosyltransferases, and hydrolases. This is one of the obstacles to designing prodrugs that are tissue-specifically activated by reductases.

Sulindac, a nonsteroidal anti-inflammatory drug, is a prodrug containing a sulfoxide moiety and is converted to sulindac sulfide, a pharmacologically active metabolite, via a reduction reaction (Duggan et al., 1977a) (Fig. 1). Since the active metabolite has a relatively long half-life in blood, the analgesic effect is sustained for a long time (Berg et al., 2013). Sulindac is unique in terms of reversible conversion from sulfide form to sulindac (Duggan et al., 1977a), which is catalyzed by flavin-containing monooxygenase 3 (Hisamuddin and Yang, 2007; Sung et al., 2020) (Fig. 1). The oxidation of sulindac to its sulfone form is catalyzed by CYP1A2, CYP1B1, and CYP3A4 (Brunell et al., 2011). The area under the curve ($AUC_{0–12\ hour}$) values of sulindac, the sulfide form, and the sulfone form upon administration of 400 mg of sulindac to healthy subjects were 16.66 ± 5.46, 20.70 ± 10.33, and 15.54 ± 4.65 μg × hr/mL, respectively (Duggan et al., 1977b). The majority of sulindac is excreted into the urine, of which approximately 30% is sulindac and approximately 50% is the sulfone form (Duggan et al., 1977b). It

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ABBREVIATIONS: AKR, aldo-keto reductase; AOX1, aldehyde oxidase 1; AUC, area under the curve; CL_{int}, intrinsic clearance; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIC, human intestinal cytosol; HIM, human intestinal microsome; HLC, human liver cytosol; HLM, human liver microsome; HRC, human renal cytosol; HRM, human renal microsome; K_m, Michaelis-Menten constantLC/MS/MS, liquid chromatography tandem mass spectrometry; MNA, *N*-methylnicotinamide; MSR, methionine sulfoxide reductase; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcription polymerase chain reaction; REF, relative expression factor; TXN, thioredoxin; T_{max}, time of maximum plasma concentration.

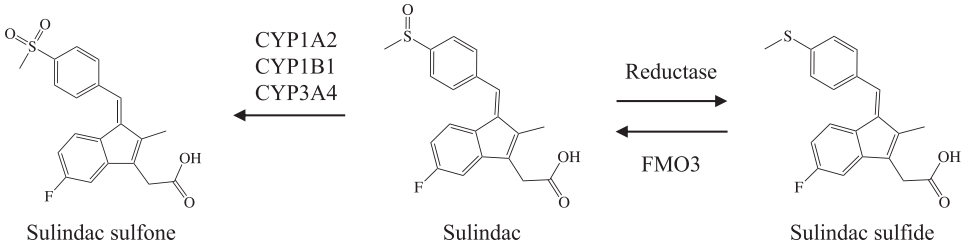

Fig. 1. Metabolic pathways of sulindac in humans.

has been reported that sulindac reduction is catalyzed by the gut microbiota (Strong et al., 1985, 1987). The production of the active form in the intestinal lumen may increase the risk of gastrointestinal injury via inhibition of cyclooxygenase 1, which plays a role in protecting the gastrointestinal mucosa (Radi and Khan, 2006). It is interesting that the frequency and severity of gastrointestinal injury induced by sulindac are lower than those of injury induced by other nonsteroidal anti-inflammatory drugs (Brogden et al., 1978). Based on this information, it is surmised that sulindac itself is absorbed from the intestine and then is efficiently converted to the active form by enzyme(s) in human tissues. It has been reported that *Escherichia (E.) coli* methionine sulfoxide reductase (Msr) catalyzes the sulindac reduction reaction (Etienne et al., 2003). The orthologous enzyme MSRA is conserved in mammals, but it remains to be studied whether mammalian MSRA catalyzes the sulindac reduction reaction. Kitamura et al. (2001) reported that monkey aldehyde oxidase 1 (AOX1) shows sulindac reductase activity, but it is unknown whether AOX1 is responsible for sulindac reduction in human tissues.

In this study, we investigated whether human tissues show sulindac reductase activity and then identified the enzyme(s) responsible for sulindac reduction in tissues. In addition, we examined whether tissues or the gut microbiota play a critical role in sulindac activation in the body.

Materials and Methods

Chemicals and Reagents. Sulindac, dithiothreitol (DTT), DMSO, purified recombinant human thioredoxin (TXN) and TXN reductase, insulin, ampicillin sodium, gentamicin sulfate, and nitrazepam were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Sulindac sulfide was purchased from Tokyo Chemical Industry (Tokyo, Japan). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Oriental Yeast (Tokyo, Japan). N^1-methylnicotinamide (MNA), metronidazole, and aminonitrazepam were purchased from Cosmo Bio (Tokyo, Japan), LKT Laboratories (Minneapolis, MN), and Cerilliant (Round Rock, TX), respectively. Human intestinal microsomes (pooled HIM, $n = 7$), human liver microsomes (pooled HLM, $n = 50$) and human liver cytosol (pooled HLC, $n = 50$) were purchased from Corning (Corning, NY). Human intestinal cytosols (pooled HIC, $n = 6$), human renal cytosols (pooled HRC, $n = 4$), and human renal microsomes (pooled HRM, $n = 8$) were purchased from XenoTech (Lenexa, KS). Purified recombinant human MSRA was purchased from Adipogen (San Diego, CA). Polyclonal rabbit anti-human MSRA (14547-1-AP), TXN (sc-20146), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NB100-56875) antibodies were purchased from Proteintech (Chicago, IL), Santa Cruz Biotechnology (Santa Cruz, CA), and IMGENEX (San Diego, CA), respectively. IRDye 680-conjugated goat anti-rabbit IgG was purchased from Li-Cor Biosciences (Lincoln, NE). Silencer Select siRNA was purchased from Life Technologies (Carlsbad, CA). All primers were commercially synthesized by Eurofins Genomics (Tokyo, Japan). All other reagents used in this study were of analytical or the highest quality grade commercially available.

Cell Culture. Human hepatocellular carcinoma-derived HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). The HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Invitrogen), 3% glutamine, 16% sodium bicarbonate, and 0.1 mM of nonessential amino acids (Invitrogen) in a 5% CO_2 atmosphere at 37°C.

Measurement of Sulindac Reductase Activity of Human Tissue Preparations and Purified Recombinant Human MSRA. Sulindac reductase activity was determined as follows: typical incubation mixtures (final volume, 0.2 mL) contained 100 mM of HEPES buffer (pH 7.4), sulindac, and enzyme sources (0.25 mg/mL pooled HIM, HIC, HLM, HLC, HRM, and HRC, or 0.75 μg/ml purified recombinant human MSRA). Sulindac was dissolved in acetonitrile, and the final concentration of acetonitrile was 1.0%. The concentration of sulindac was 20 μM. The reaction was initiated by the addition of an reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (0.5 mM of $NADP^+$, 5 mM of glucose-6-phosphate, 5 mM of $MgCl_2$, and 1 U/ml of glucose-6-phosphate dehydrogenase), MNA, or DTT after a 2-minute preincubation at 37°C. NADPH, MNA, and DTT were used as electron donors for AKR (Penning, 2015)/short-chain dehydrogenase/reductase (Ahmed et al., 1979), AOX1 (Wolpert et al., 1973), and reductases, respectively, depending on the thioredoxin system (Lowther et al., 2000). After a 30-minute (purified recombinant MSRA) or 60-minute (tissue preparations) incubation at 37°C, the reaction was terminated by the addition of 100 μl of ice-cold acetonitrile. The protein concentration and incubation time were determined by confirming the linearity of sulindac sulfide formation up to 0.5 mg/ml (tissue preparations) and 1.5 μg/ml (purified recombinant MSRA) and 90 minutes. After removing the protein by centrifugation at 20,380 g for 5 minutes, a 10-μl aliquot of the supernatant was subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS). An LCMS-8045 liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an LC-20AD high-performance liquid chromatography system was used. The LC instrument comprised a CBM-20A controller (Shimadzu), LC-20AD pumps (Shimadzu), an SIL-20AC HT autosampler (Shimadzu), a CTO-20AC column oven (Shimadzu), and an SPD-20A UV detector (Shimadzu) equipped with a Develosil ODS-UG-3 column (3 μm particle size, 4.6 mm i.d. × 150 mm; Nomura Chemical, Seto, Japan). The column temperature was set at 40°C, and the flow rate was 0.2 ml/min. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The conditions for elution were as follows: 30–90% B (0–2 minutes), 90% B (2–8 minutes), and 30% B (8–12 minutes). LC/MS/MS was performed in positive electrospray mode. Nitrogen was used as the nebulizing and drying gas at rates of 3 L/min and 15 L/min, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using argon as the collision gas at 230 kPa. Sulindac sulfide was monitored in multiple reaction monitoring (MRM) mode at *m/z* values of 340.90 and 233.05. The collision energy was -47 V. The analytical data were processed using LabSolutions (version 5.82 SP1, Shimadzu).

Kinetic Analysis. Kinetic analyses were performed using pooled HIC, HLC, HRC, and purified recombinant human MSRA with substrate concentrations ranging from 10 to 250 μM. The kinetic parameters were determined from the Lineweaver-Burk plot.

Analysis of Sulindac Reductase Activity Inhibition. The inhibitory effects of DMSO, an inhibitor of mammalian MsrA (Kwak et al., 2009), on the sulindac reductase activity of HIC, HLC, HRC, and purified recombinant human MSRA were examined. The concentration of DMSO ranged from 0.01 to 1%. The experimental procedure and conditions were the same as those described above except that 10 mM of DTT was present in the incubation mixtures.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of MSRA and TXN were performed. For analysis of MSRA expression, human tissue cytosol (20 μg) or cell homogenates (40 μg) or purified recombinant human MSRA (35 ng) was separated on 15% polyacrylamide gels. For analysis of TXN expression, human tissue cytosol (30 μg) and recombinant human TXN (0.4 μg) were separated on 15% polyacrylamide gels. For analysis

of GAPDH expression, cell homogenates (3 μg) was separated on 10% polyacrylamide gels. The proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA). The membranes were probed with the primary antibody and then with the fluorescent dye-conjugated secondary antibody. The band intensities were quantified using an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE). The MSRA protein level in HepG2 cells was normalized to the GAPDH protein level.

Contribution of MSRA to Sulindac Reductase Activity in Human Tissue Cytosol. The contribution of MSRA to sulindac reductase activity in human tissue cytosols was calculated by applying the relative expression factor (REF). The REF of MSRA (REF_{MSRA}) was determined as the ratio of MSRA protein abundance in the tissue cytosol to the abundance of the recombinant enzyme according to the band intensity relative to the amount of protein in micrograms determined by immunoblot analysis. Using the REF and sulindac reductase activity of purified recombinant human MSRA (V_{rMSRA}), the activity of tissue MSRA ($V_{tissue\ MSRA}$) was estimated as follows:

$$V_{\text{tissue MSRA}} = V_{\text{rMSRA}}' \text{REF}_{\text{MSRA}} \quad (1)$$

The contribution of MSRA to sulindac reductase activity in the tissue cytosol ($V_{tissue\ cytosol}$) was calculated using the following equation:

$$\text{Contribution of MSRA } (\%) = \left(V_{\text{tissue MSRA}} / V_{\text{tissue cytosol}}\right)' 100 \quad (2)$$

Based on the fact that cytosolic factor(s) increased the sulindac reductase activity of purified recombinant MSRA, the sulindac reductase activity of purified recombinant MSRA in the presence of cytosol ($V_{rMSRA+cytosol}$) was calculated as follows:

$$V_{\text{rMSRA+cytosol}} = (P_{\text{rMSRA+cytosol}} - P_{\text{cytosol}})/\text{incubation time/protein amount of purified recombinant MSRA} \quad (3)$$

where $P_{rMSRA+cytosol}$ and $P_{cytosol}$ are the amounts of sulindac sulfide produced by the "combination of purified recombinant MSRA and cytosol" and "only cytosol", respectively. The amounts of sulindac sulfide produced by the combination of purified recombinant MSRA and human tissue cytosols (HIC, HLC, or HRC) were quantified as described above. The activity of MSRA in tissue under activation by cytosolic factor(s) ($V_{tissue\ MSRA+cytosol}$) was estimated using the REF as follows:

$$V_{\text{tissue MSRA+cytosol}} = V_{\text{rMSRA+cytosol}}\ ' \text{REF}_{\text{MSRA}} \quad (4)$$

The contribution of MSRA to sulindac reductase activity under activation by cytosolic factor(s) in the cytosol was calculated using the following equations:

$$\text{Contribution of MSRA } (\%) = \left(V_{\text{tissue MSRA+cytosol}} / V_{\text{cytosol}}\right)' 100 \quad (5)$$

Measurement of Insulin Reductase Activity. TXN activity was determined by measuring insulin reductase activity according to a previous report (Lundstrom and Holmgren, 1990) with slight modifications. The incubation mixture (final volume, 0.2 ml) contained 100 mM of HEPES buffer (pH 7.4), 1 mM of EDTA, an NADPH-generating system, 300 μM of insulin, and enzyme sources (50 μg/ml of pooled HIC, HLC, and HRC and 4 μg/mL of purified recombinant human TXN). The reaction was started by the addition of recombinant human TXN reductase. After a 30-minute incubation at 37°C, the reaction was terminated by the addition of 200 μl of 6.4 M guanidine HCl, 100 mM of Tris-HCl (pH 8.0), and 5 mM of 5,5′-dithiobis(2-nitrobenzoic acid). The concentration of reduced insulin with a thiol group was determined by measuring the absorbance at 412 nm. The insulin reductase activity of recombinant TXN was defined as 1 AU.

Knockdown of MSRA and Measurement of Sulindac Reductase Activity in HepG2 Cells. HepG2 cells were seeded in 48-well plates (6×10^4 cells/well), 24-well plates (1.5×10^5 cells/well), or 6-well plates (6×10^5 cells/well) and transfected with 10 nM of Silencer Select siRNA using Lipofectamine RNAiMAX (Life Technologies). After 48 hours, the cells were incubated with culture medium containing 20 μM of sulindac for 5 hours at 37°C, and 150 μl of acetonitrile was added to 150 μl of the collected medium. After centrifugation at 20,380 g for 5 minutes, a 10-μl aliquot of the supernatant was subjected to LC/MS/MS. The amount of sulindac sulfide was measured according to the method described above. Knockdown of MSRA was confirmed by quantitative reverse-transcription polymerase chain reaction (qRT–PCR) and immunoblot analysis. For qRT–PCR, total RNA was prepared using RNAiso (Takara Bio, Shiga, Japan) according to the manufacturer's protocols. For immunoblot analysis, cells were collected, suspended in TGE buffer (10 mM of Tris-HCl, 20% glycerol, and 1 mM of EDTA, pH 7.4), and disrupted by freezing and thawing three times. Then, the suspensions were homogenized by ten strokes with a Teflon-glass homogenizer.

qRT–PCR Analysis of MSRA mRNA Expression. cDNA was synthesized from total RNA using ReverTra Ace. A 1-μl aliquot of the reverse-transcribed mixture was added to a polymerase chain reaction (PCR) mixture containing 5 pmol of each primer and 10 μl of Luna Universal qPCR mix in a final volume of 20 μl. A forward human MRSA primer (5′- GCC ATC TAC CCG ACC TCT GC -3′) and reverse human MRSA primer (5′- AGG CCG CAG TAG CCA TTG -3′) were used, and a forward mouse MsrA primer (5′- TAT TTG GAA TGG GCT GCT TC -3′) and reverse mouse MsrA primer (5′- GTA GGT GGG ATT GCG TGT G -3′) were used. The PCR conditions for human MSRA were as follows: after an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 95°C for 15 s, followed by annealing/extension at 58°C for 30 s for 40 cycles. The PCR conditions for mouse MSRA were as follows: after an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 95°C for 15 s, followed by annealing/extension at 55°C for 30 s for 40 cycles. Quantitative PCR was performed using QuantStudio 1 (Thermo Fisher Scientific). The sequences of the primers and PCR conditions for human and mouse GAPDH were previously described (Tsuchiya *et al.*, 2004; Kobayashi *et al.*, 2010). The human MSRA and mouse MsrA mRNA levels were normalized to the human GAPDH and mouse Gapdh mRNA levels, respectively.

Animals. C57BL/6J mice were housed in the institutional animal facility in a controlled environment (temperature 25 ± 1°C and 12 hour light/dark cycle) with ad libitum access to food and water according to the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Preparation of Mouse Tissue Cytosols and Measurement of the Sulindac Reductase Activity. Pooled mouse intestinal, liver, or renal cytosols were prepared from mice (9–10 weeks old, male, $n = 6$) according to our previous report (Sakai et al., 2021). Sulindac reductase activity was determined as described above with 0.2 mg/ml of mouse tissue cytosols after incubation for 60 minutes at 37°C based on the linearity of sulindac sulfide formation up to 0.5 mg/ml and 90 minutes, respectively. The effects of DMSO (0.01–1%) and antibiotics (ampicillin, metronidazole, and gentamicin) (1 mM) on the sulindac reductase activity of mouse tissue cytosols were also evaluated.

Antibiotic Treatment and Measurement of Plasma Concentrations of Sulindac and Sulindac Sulfide. Mice (8–10 weeks old, male, body weight: 25.9 ± 3.2 g) received oral treatment with a broad-spectrum antibiotic cocktail (ampicillin, metronidazole: 100 mg/kg, and gentamycin: 5 mg/kg dissolved in saline) once a day for 3 days according to a previously reported method (Sun et al., 2019) with slight modifications. RNA from the intestines, livers, and kidneys of mice sacrificed 24 hours after the last administration was isolated using RNAiso according to the manufacturer's protocol. Sulindac (8 mg/kg dissolved in 1% carboxymethyl cellulose, *p.o.*) was administered to the mice 24 hours after 3 days of antibiotic treatment. Blood was collected from the tail vein at 0.33, 0.67, 1, 2, 4, 6, 9, 12, and 24 hours, and the plasma concentrations of sulindac and sulindac sulfide were measured as follows: 5 μl of plasma sample, 45 μ of acetonitrile, and 50 μl of Milli-Q water were vigorously mixed for 1 minute and centrifuged at 20,380 g for 5 minutes. Ten microliters of the supernatant were subjected to LC/MS/MS. The apparatus and conditions were the same as described above. Sulindac sulfide levels were monitored as described above, sulindac levels were analyzed in MRM positive mode at *m/z* values of 357.10 and 233.00 and a collision energy of -50 V.

Measurement of Plasma Concentrations of Nitrazepam and Aminonitrazepam. Twenty-four hours after 3 days of antibiotic treatment, nitrazepam (100 mg/kg dissolved in corn oil, *p.o.*) was administered to the mice (8–10 weeks old, male, body weight: 25.0 ± 1.9 g). Blood was collected from the tail vein at 1, 3, 6, 9, 12, and 24 hours, and plasma concentrations of nitrazepam and aminonitrazepam were measured as follows: 2 μl of plasma sample, 48 μl of acetonitrile, and 50 μl of Milli-Q water were vigorously mixed for 1 minute and centrifuged at 20,380 g for 5 minutes. Five microliters of the supernatant were subjected to LC/MS/MS. The concentrations of nitrazepam and aminonitrazepam were measured according to a previously reported method (Konishi et al., 2017).

Statistical Analysis. Statistical significance between two groups was determined by Student's *t test*. $P < 0.05$ was considered significant.

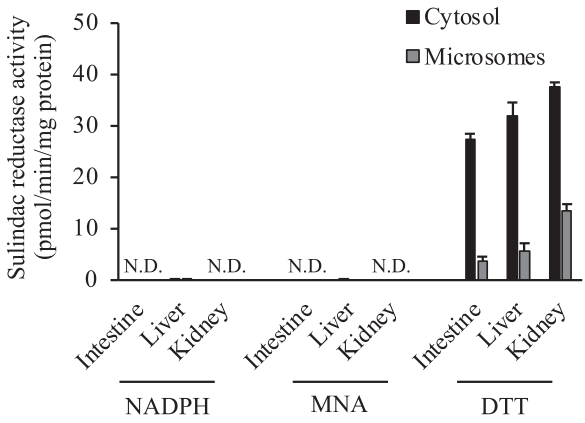

Fig. 2. Sulindac reductase activity in the cytosol and microsomes from the human intestine, liver, and kidney. Human tissue fractions (0.25 mg/ml) were incubated with 20 μM of sulindac for 60 minutes in the presence of an NADPH-generating system, 1 mM of MNA, or 10 mM of DTT. Each column represents the mean ± SD of three experiments.

Results

Sulindac is Converted to Sulindac Sulfide in Human Tissues. In this study, the sulindac reductase activity of pooled HIM, HIC, HLM, HLC, HRM, and HRC was evaluated at a substrate concentration of 20 μM (Fig. 2), which is the maximum plasma concentration (C_{max}) achieved upon administration of 200 mg sulindac to healthy subjects (Tang et al., 2017). In the absence of electron donors (data not shown), sulindac reductase activity was not observed. Similarly, sulindac reductase activity was hardly observed in the presence of an NADPH-generating system or MNA, indicating no contribution of major drug-metabolizing reductases, such as AKR, CBR, or AOX1 to sulindac reduction. In the presence of DTT, the sulindac reductase activity of HIC, HLC, and HRC was 27.4 ± 1.0, 31.8 ± 2.7, and 37.5 ± 0.9 pmol/min/mg protein, respectively, and HIM, HLM, and HRM showed approximately 1/8–1/3 of the activity of the corresponding cytosolic fractions. Because DTT slightly reduced sulindac to sulindac sulfide even in the absence of the enzyme sources (data not shown), the activity values described above were calculated by subtracting the amount of sulindac sulfide formed by DTT. Thus, it was demonstrated that human tissues can convert sulindac to its sulfide form and that the responsible enzyme is mainly localized in the cytosol and possibly depends on the thioredoxin system.

Human MSRA is Responsible for Sulindac Reduction. It has been reported that sulindac is reduced by Msr in *E. coli* (Etienne et al., 2003). To investigate the involvement of the orthologous human enzyme MSRA, which is localized in the tissue cytosol and exerts activity depending on the thioredoxin system (Lowther et al., 2000), in the reduction reaction of sulindac, the reductase activity of purified recombinant human MSRA was measured (Fig. 3A). In the absence of electron donors (data not shown) or the presence of an NADPH-generating system or MNA, purified recombinant MSRA was not observed to have sulindac reductase activity, whereas in the presence of DTT, sulindac reductase activity of purified recombinant MSRA was observed (7.7 ± 1.3 nmol/min/mg protein). Thus, these results suggested that the sulindac reductase activity of human tissue cytosols may be due to MSRA.

Kinetic analysis of sulindac reduction was performed using pooled HIC, HLC, HRC, and purified recombinant MSRA in the presence of 10 mM of DTT (Fig. 3, B and C). The sulindac reductase activity of tissue cytosols and purified recombinant MSRA was decreased at high sulindac concentrations, but it did not fit the substrate inhibition equation. The kinetic parameters were calculated from the Lineweaver-Burk plot (Supplemental Fig. 1), and the Michaelis constant (K_m) values of

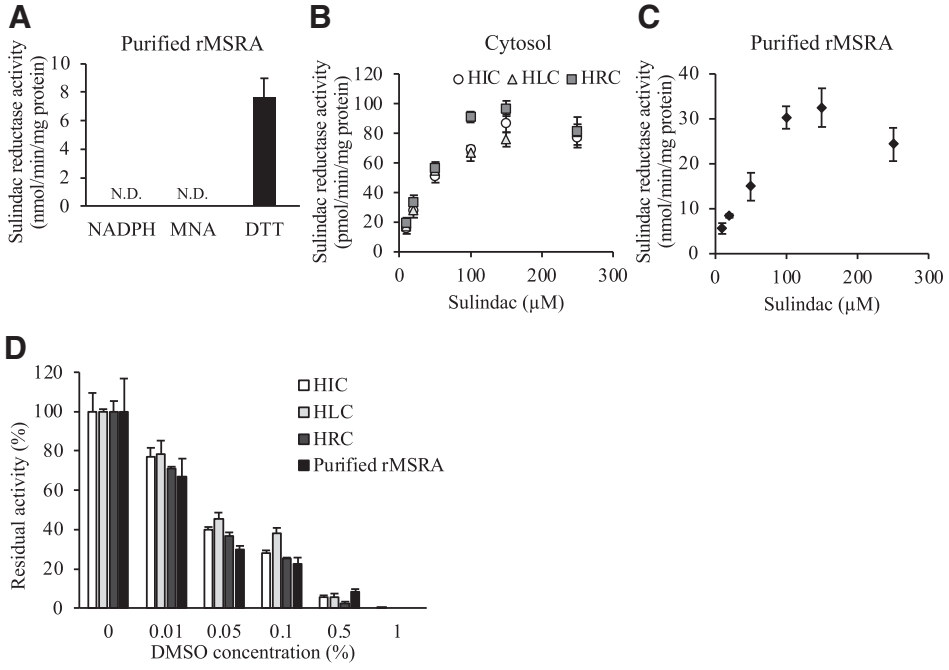

Fig. 3. Sulindac reduction by human MSRA. (A) Sulindac reductase activity of purified recombinant human MSRA. Purified recombinant human MSRA (0.75 μg/ml) was incubated with 20 μM sulindac for 30 minutes in the presence of an NADPH-generating system, 1 mM of MNA, or 10 mM DTT. Each column represents the mean ± SD of three experiments. B, C Kinetic analysis of sulindac reductase activity of human tissue cytosols (B) and purified recombinant human MSRA (C). Human tissue cytosols (0.25 mg/ml) and purified recombinant human MSRA (0.75 μg/ml) were incubated with 20 μM of sulindac for 30 minutes (human tissue cytosols) or 60 minutes (purified recombinant human MSRA) in the presence of 10 mM of DTT. Each data point represents the mean ± SD of three experiments. (D) Effects of DMSO on the sulindac reductase activity of HIC, HLC, HRC, and purified recombinant human MSRA. Human tissue cytosols (0.25 mg/ml) and purified recombinant human MSRA (0.75 μg/ml) were incubated with 20 μM sulindac for 30 minutes (human tissue cytosols) or 60 minutes (purified recombinant human MSRA) in the presence of 10 mM DTT. The concentrations of DMSO were 0.01–1%. Each column represents the mean ± SD of three experiment.

TABLE 1

Kinetic parameters of the sulindac reductase activity of human tissue cytosols and purified recombinant MSRA. All data are expressed as the mean ± SD.

	K_m(μM)	V_{max}(pmol/min/mg protein)	CL_{int}(μL/min/mg protein)
HIC	62.3 ± 4.8	111.6 ± 3.1	1.79 ± 0.09
HLC	50.9 ± 7.9	101.2 ± 11.0	2.00 ± 0.11
HRC	53.1 ± 17.2	119.0 ± 12.8	2.34 ± 0.48
Purified recombinant MSRA	59.2 ± 22.9	35703.3 ± 7219.4	641.83 ± 150.62

CL_{int}, intrinsic clearance; K_m, Michaelis constant; V_{max}, maximum velocity

cytosolic fractions (HIC, 62.3 ± 4.8; HLC, 50.9 ± 7.9; HRC, 53.1 ± 17.2 μM) were similar to that of purified recombinant MSRA (59.2 ± 22.9 μM) (Table 1). Thus, similar characteristics between tissue cytosols and purified recombinant MSRA suggested that MSRA is an enzyme responsible for sulindac reduction in tissue cytosols.

Since it has been reported that mouse MsrA is potently inhibited by DMSO (Kwak et al., 2009), the effects of DMSO on the sulindac reductase activity of human tissue cytosols and purified recombinant MSRA were examined. As shown in Fig. 3D, the sulindac reductase activity of HIC, HLC, and HRC was potently inhibited by DMSO in a concentration-dependent manner. In addition, similar inhibitory potency was observed when purified recombinant MSRA was used as an enzyme source. These results indicate that the enzyme responsible for sulindac reduction in human tissues is MSRA.

Contribution of MSRA to Sulindac Reductase Activity in Human Tissue Cytosol. The contribution of MSRA to sulindac reductase activity in human cytosols was calculated using REF (Table 2). According to immunoblot analysis, the REF values of HIC, HLC, and HRC were 6.7×10^{-4}, 4.6×10^{-4}, and 7.3×10^{-4}, respectively (Fig. 4A). The contribution percentages of MSRA calculated using these REF values were 20.6%, 12.9%, and 13.8% in HIC, HLC, and HRC, respectively (Table 2). Considering the possibility that some factors in the cytosol enhance the activity of MSRA, whether the production of sulindac sulfide by purified recombinant MSRA is synergistically increased by the addition of tissue cytosol was investigated (Fig. 4B). When purified recombinant MSRA (0.75 μg/ml) and human tissue cytosols (0.25 mg/ml) were incubated with 20 μM of sulindac for 30 minutes, the amount of sulindac sulfide produced by purified recombinant MSRA, HIC, HLC, and HRC was 33.1 ± 1.7, 36.1 ± 0.4, 38.8 ± 3.6, and 58.7 ± 2.8 pmol, respectively. The amount of sulindac sulfide produced by purified recombinant MSRA was synergistically increased by the addition of HIC (246.3 ± 13.8 pmol), HLC (303.9 ± 31.4 pmol), or HRC (283.7 ± 18.4 pmol). These results suggest that some factors in the cytosolic fraction enhance the activity of MSRA. Under this condition, the contribution percentage of MSRA was calculated to be 130.1%, 104.7%, and 93.4% in HIC, HLC, and HRC, respectively (Table 3), suggesting that MSRA is a major enzyme that catalyzes sulindac reduction in human tissues.

TXN is Not a Principal Factor in Increasing MSRA Enzyme Activity. Since it has been reported that TXN activates MSRA via thiol-disulfide exchange in mammals (Lowther et al., 2000), we investigated whether TXN is a cytosolic factor that enhances MSRA activity. As shown in Fig. 4C, the sulindac reductase activity of purified recombinant MSRA was increased by the addition of recombinant TXN in a concentration-dependent manner. According to immunoblot analysis, the protein abundance of TXN in HIC, HLC, and HRC was calculated to be 1.8×10^{-3}, 0.6×10^{-3}, and 0.4×10^{-3} unit/μg, respectively, with reference to the band intensity per microgram of recombinant TXN as 1 unit/μg (Fig. 4D). Although the TXN level in HIC, HLC, and HRC was approximately 1,000-fold lower than that in recombinant TXN (Fig. 4D), the rates of the increase induced by 250 μg/ml of tissue cytosols (HIC, 6.4-fold; HLC, 8.0-fold; HRC, 6.8-fold; Fig. 4B) were almost equal to those induced by 25 μg/ml of recombinant TXN (5.6-fold) (Fig. 4C). To investigate the possibility that the specific activity of TXN was different between tissue cytosols and recombinant TXN, insulin reductase activity was measured. The results showed that the activity of HIC, HLC, and HRC (0.6×10^{-2}, 0.5×10^{-2}, and 1.0×10^{-2} AU, respectively) was approximately 100-fold lower than the activity of recombinant TXN (1.0 ± 0.1 AU) (Fig. 4E). The inconsistency of differences in TXN abundance (1,000-fold) and specific activity (100-fold) between tissue cytosols and recombinant TXN may be attributed to the difference in the oxidation state of TXN in human tissue cytosols and recombinant TXN because DTT was not included in the incubation mixture when insulin reductase activity was measured. For the estimation methods using the protein level and the specific activity of TXN, the contribution of TXN to the enhancement of MSRA-catalyzing sulindac reduction activity in tissue cytosols was quite low (1–10%). Thus, TXN is not a principal factor in increasing MSRA enzyme activity.

MSRA is an Enzyme Responsible for Sulindac Reduction in Human Cells in which the Thioredoxin System is Present. To confirm whether MSRA is the enzyme responsible for sulindac reduction in human cells, the effects of knockdown of MSRA on sulindac reductase activity in HepG2 cells were examined (Fig. 5). It was confirmed that siMSRA treatment effectively decreased MSRA expression at the mRNA and protein levels (Fig. 5, A and B). Sulindac reductase activity was significantly decreased from 28.7 ± 2.8 to 13.6 ± 2.1 pmol/h/mg protein by knockdown of MSRA (Fig. 5C). This result suggests that MSRA is the enzyme responsible for sulindac reduction in living human cells in which the thioredoxin system is present.

Tissue MsrA Activates Sulindac, Making a Greater Contribution than the Gut Microbiota in Mice. We sought to evaluate the contribution of tissue MsrA and the gut microbiota to sulindac activation in mice in vivo. Prior to the in vivo experiment using mice, to elucidate whether sulindac could be reduced by mouse MsrA as well as human MSRA, the inhibitory effect of DMSO on sulindac reductase activity in mouse tissue cytosols was examined at a substrate concentration of 20 μM (Fig. 6A). In the presence of DTT, sulindac reductase activity in mouse intestinal, liver, and renal cytosols (14.0 ± 0.2, 41.4 ± 1.3, and 34.3 ± 1.5 pmol/min/mg protein, respectively) was almost completely inhibited by DMSO. These results suggest that sulindac could also be reduced by MsrA in mice.

TABLE 2

Contribution of MSRA to sulindac reductase activity in the human tissue cytosol. All data are expressed as the mean ± SD.

	$V_{cytosol}$(pmol/min/mg protein)	$V_{tissue\ MSRA}$(pmol/min/mg protein)	Contribution of MSRA (%)
HIC	24.1 ± 0.3	5.0	20.6
HLC	25.9 ± 2.4	3.4	12.9
HRC	39.1 ± 1.8	5.4	13.8

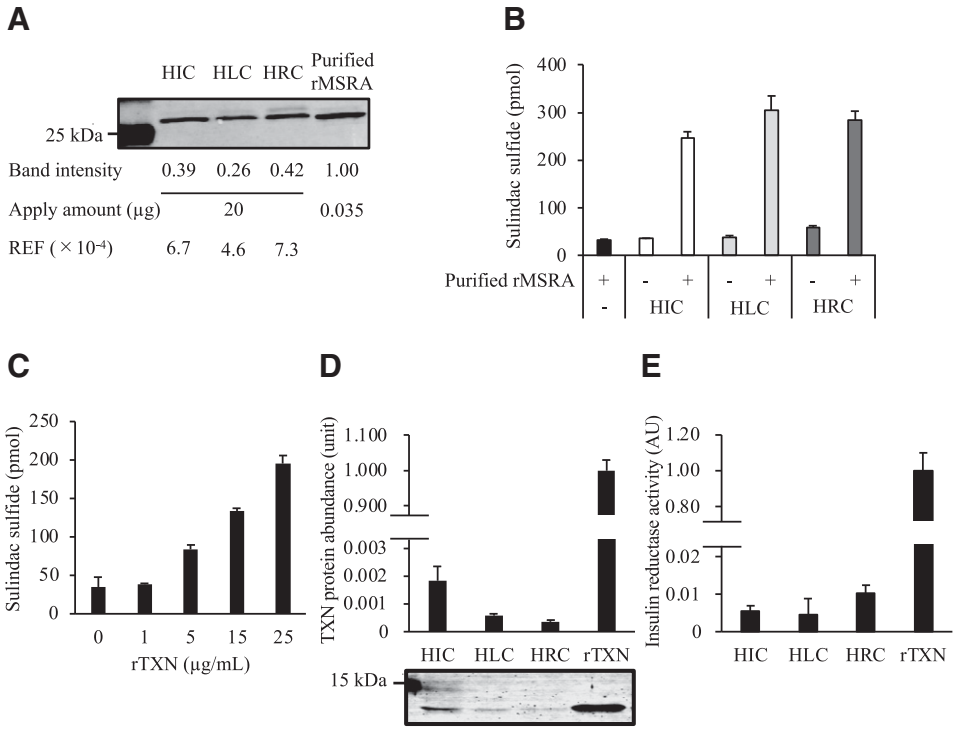

Fig. 4. Contribution of MSRA to sulindac reductase activity in human tissue cytosol. (A) Relative MSRA protein abundance in HIC, HLC, HRC, and purified recombinant human MSRA. HIC, HLC, and HRC (20 μg) and purified recombinant human MSRA (0.035 μg) was separated by electrophoresis using a 15% SDS-polyacrylamide gel. (B) The amount of sulindac sulfide produced by purified recombinant human MSRA in the absence and presence of tissue cytosols. Human tissue cytosols (0.25 mg/ml) and purified recombinant human MSRA (0.75 μg/ml) were incubated with 20 μM of sulindac for 30 minutes in the presence of 10 mM of DTT. Each column represents the mean ± SD of three experiments. (C) Effects of TXN on the sulindac reductase activity of purified recombinant human MSRA. Purified recombinant human MSRA (0.75 μg/ml) was incubated with 20 μM sulindac for 30 minutes in the presence of 10 mM of DTT and recombinant TXN. D, E Relative TXN protein abundance (D) and activity (E) in HIC, HLC, HRC, and recombinant TXN. HIC, HLC, HRC (50 μg/ml), or recombinant human TXN (4 μg/ml) were incubated with 300 μM of insulin for 30 minutes in the presence of an NADPH-generating system and recombinant human TXN reductase. HIC, HLC, and HRC (30 μg) and recombinant TXN (0.4 μg) was separated by electrophoresis using a 15% SDS-polyacrylamide gel. Each column represents the mean ± SD of three experiments.

To examine the contribution of tissue MsrA, the mouse gut microbiota was depleted by oral administration of antibiotics. To confirm the elimination of the gut microbiota, the pharmacokinetics of nitrazepam, which has been reported to be reduced to aminonitrazepam by the gut microbiota in rats (Takeno and Sakai, 1991), were evaluated (Fig. 6B). The AUC of nitrazepam (442.6 ± 83.1 μg × hr/ml) was significantly increased (751.8 ± 140.0 μg × hr/ml) and that of aminonitrazepam (53.3 ± 16.5 μg × hr/ml) was substantially decreased by preadministration of antibiotics (2.6 ± 0.6 μg × hr/ml) (Fig. 6B). MsrA mRNA levels in mouse tissues were not significantly changed by antibiotic treatment (Fig. 6C). In addition, an in vitro study confirmed that sulindac reductase activity in mouse tissue cytosols was not significantly changed by the antibiotics used in this study (Fig. 6D). Thus, the antibiotics per se did not alter the activity and expression of MsrA. In mice orally administered 8 mg/kg of sulindac, the C_{max} (29.5 ± 10.2 μM) and AUC (28.4 ± 2.2 μg × hr/ml) of sulindac in antibiotic-preadministered mice were significantly lower than those (C_{max}, 51.0 ± 11.3 μM; AUC, 66.1 ± 15.7 μg × hr/ml) in vehicle-preadministered mice. (Fig. 6, E and F, Table 4). In addition, the time of maximum plasma concentration (T_{max}) of both sulindac (0.60 ± 0.28 hours) and sulindac sulfide (2.20 ± 1.10 hours) was shortened by preadministration of antibiotics (sulindac, 0.39 ± 0.14 hour; sulindac sulfide, 0.72 ± 0.25 hours) (Fig. 6, E and F, Table 4). These results suggest that the absorption of sulindac may be inhibited by antibiotics. The contribution of tissue MsrA to sulindac activation was estimated by the ratio of AUC values of sulindac sulfide and sulindac. The AUC ratio (1.29) was decreased by 31% by preadministration of antibiotics (0.89) (Table 4). Thus, the contribution of MsrA in mouse tissues to sulindac activation is expected to be 69% in the body. These results indicate that MsrA in tissues rather than the gut microbiota plays a critical role in sulindac activation in the body.

Discussion

Sulindac, a prodrug containing a sulfoxide moiety, is converted to sulindac sulfide, the pharmacologically active metabolite, by a reduction reaction (Duggan et al., 1977a). The reduction of sulindac has been

TABLE 3

Contribution of MSRA to sulindac reductase activity in human tissue cytosol under activation by cytosolic factor(s).
All data are expressed as the mean ± SD.

	$P_{rMSRA+cytosol}$ (pmol)	$P_{cytosol}$ (pmol)	$V_{rMSRA+cytosol}$ (nmol/min/mg protein)	$V_{tissue\ MSRA+cytosol}$ (pmol/min/mg protein)	Contribution of MSRA (%)
HIC	246.3 ± 13.8	36.1 ± 0.4	46.7	31.3	130.1
HLC	303.9 ± 31.4	38.8 ± 3.6	58.9	27.1	104.7
HRC	283.7 ± 18.4	58.7 ± 2.8	50.0	36.5	93.4

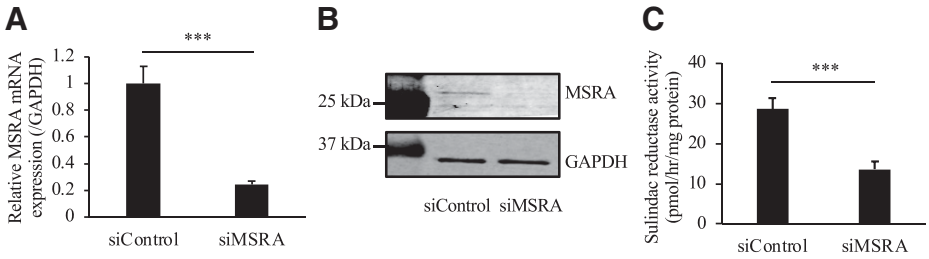

Fig. 5. Effects of MSRA knockdown on sulindac reductase activity in HepG2 cells. Relative MSRA mRNA (A) and protein (B) levels and sulindac reductase activity (C) were measured 48 hours after the transfection of 10 nM of siRNA targeting MSRA (siMSRA) or control (siControl). Cell homogenates (MSRA: 40 μg, GAPDH: 3 μg) were separated by electrophoresis using a 15% SDS-polyacrylamide gel. Each column represents the mean ± SD (n = 4). ***P < 0.001.

reported to be due to the gut microbiota (Strong et al., 1985, 1987). However, the low frequency of gastrointestinal injury caused by sulindac made us surmise that enzyme(s) in tissues rather than the gut microbiota efficiently catalyze the sulindac reduction reaction, allowing it to exert its pharmacological effect. In this study, we sought to identify the enzyme(s) responsible for sulindac reduction in tissues and to evaluate its/their contribution to sulindac activation in the body.

Higher sulindac reductase activity was observed in the HIC, HLC, and HRC than in microsomal fractions in the presence of DTT (Fig. 2), revealing that enzyme(s) in human tissue cytosols can reduce sulindac via thiol-disulfide exchange. A group of redox proteins, such as thioredoxin and glutaredoxin, catalyze a series of thiol-disulfide exchange reactions and activate various reductases (Holmgren, 1989). In vitro, DTT is frequently used in place of the thioredoxin system (Lowther et al., 2000). Kitamura et al. (2001) reported that monkey liver cytosol shows sulindac reductase activity at a high substrate concentration (500 μM) in the presence of 2-hydroxypyrimidine, which is oxidized by AOX1 via transfer of two electrons to AOX1 (Wolpert et al., 1973). However, in our study, sulindac reductase activity was hardly observed in human tissue cytosol at a substrate concentration of 20 μM in the presence of MNA, which is an electron donor of AOX1 as well as 2-hydroxypyrimidine (Wolpert et al., 1973) (Fig. 2). Although slight activity was observed in the presence of MNA at a substrate concentration of 100 μM (data not shown), it is believed that the contribution of AOX1 to sulindac reduction in human tissues is quite low in clinical situations. In addition, consistent with the fact that there are no reports of sulfoxide reduction by AKR or short-chain dehydrogenase/reductase, sulindac reductase activity was hardly observed in the presence of NADPH.

Since sulindac is known to be reduced by MsrA in *E. coli* (Etienne et al., 2003), we investigated whether human ortholog MSRA, which is localized in tissue cytosol and exerts its activity in a manner dependent

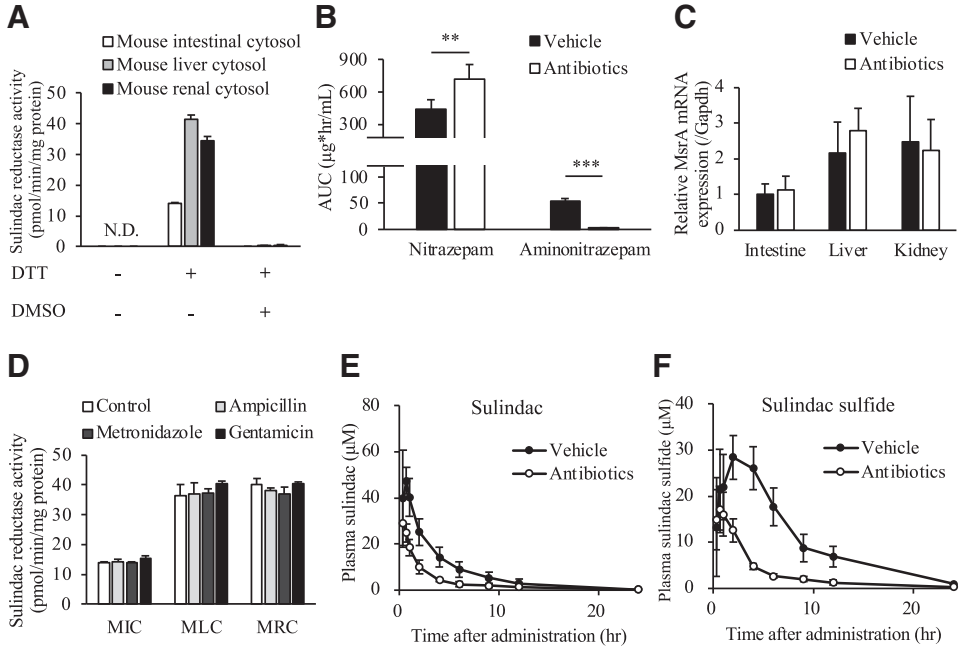

Fig. 6. Effects of antibiotic treatment on the pharmacokinetics of sulindac and sulindac sulfide in mice orally administered sulindac. (A) Effects of DMSO on sulindac reductase activity in mouse tissue cytosols. Mouse tissue cytosols (0.2 mg/ml) were incubated with 20 μM of sulindac for 60 minutes in the absence or presence of 10 mM of DTT. The concentration of DMSO was 1%. Each column represents the mean ± SD of three experiments. (B) AUC of nitrazepam and aminonitrazepam. The plasma concentrations of nitrazepam and aminonitrazepam were measured 1, 3, 6, 9, 12, and 24 hours after administration of nitrazepam (100 mg/kg dissolved in corn oil, *p.o.*) following preadministration of antibiotic cocktail or vehicle. Each column represents the mean ± SD (n = 6). **P < 0.01, ***P < 0.001. (C) Effect of antibiotic treatment on MsrA mRNA expression levels in mouse tissues. Each column represents the mean ± SD (n = 6). (D) Effects of antibiotic treatment on sulindac reductase activity in mouse tissue cytosols. Mouse tissue cytosols (0.2 mg/ml) were incubated with 20 μM of sulindac for 60 minutes in the presence of 10 mM of DTT and antibiotics. Each column represents the mean ± SD of three experiments. E, F The plasma concentrations of sulindac (E) and sulindac sulfide (F) were measured 0.33, 0.67, 1, 2, 4, 6, 9, 12, and 24 hours after administration of sulindac (8 mg/kg dissolved in 1% carboxymethyl cellulose, *p.o.*) following preadministration of antibiotic cocktail or vehicle. Each data point represents the mean ± SD (n = 5–6).

TABLE 4

Pharmacokinetic parameters of plasma sulindac and sulindac sulfide in mice orally administered 8 mg/kg of sulindac.
All data are expressed as the mean ± SD. **P < 0.01 and ***P < 0.001, compared with vehicle-treated mice (Student's t test).

	Sulindac			Sulindac sulfide			
	C_{max}(μM)	T_{max}(hr)	AUC(μg × hr/mL)	C_{max}(μM)	T_{max}(hr)	AUC(μg × hr/mL)	AUC ratio(sulindac sulfide/sulindac)
Vehicle	51.0 ± 11.3	0.60 ± 0.28	66.1 ± 15.7	30.4 ± 3.9	2.20 ± 1.10	85.1 ± 15.2	1.29
Antibiotics	29.5 ± 10.2**	0.39 ± 0.14	28.4 ± 2.2***	18.0 ± 5.1***	0.72 ± 0.25**	25.2 ± 2.1***	0.89

on the thioredoxin system (Lowther et al., 2000) (Fig. 7), catalyzes the sulindac reduction reaction. Purified recombinant human MSRA showed sulindac reductase activity with a similar K_m value as tissue cytosols (Fig. 3, A–C, Table 1). It has been reported that mouse MsrA is potently inhibited by DMSO (Kwak et al., 2009). Since it was demonstrated that DMSO treatment results in the accumulation of *S*-methionine sulfoxide, an endogenous MSRA substrate, in human SK-Hep1 cells (Kwak et al., 2009), it is believed that DMSO may inhibit human MSRA as well as mouse MsrA. As expected, DMSO inhibited sulindac reductase activity by purified recombinant human MSRA. In addition, the activity of tissue cytosols was potently inhibited by DMSO (Fig. 3D). The contribution of MSRA to sulindac reductase activity in human tissue cytosols was calculated to be almost 100% in all tissues by accounting for the increase in MSRA activity by cytosolic factor(s) (Fig. 4B, Table 3). These results suggested that MSRA is responsible for sulindac reduction in human tissues. In addition, knockdown of MSRA resulted in a significant decrease in sulindac reductase activity in HepG2 cells, revealing the role of MSRA in sulindac reduction in living human cells in which the thioredoxin system is present (Fig. 5). It is reasonable that sulindac reductase activity occurs in tissue cytosols because MSRA is mainly localized in the cytosol (Kim and Gladyshev, 2006), but such activity was also observed in microsomal fractions. The sulindac reductase activity of human tissue microsomes (Fig. 2) was also potently inhibited by DMSO (data not shown). Therefore, the reduction reaction in microsomal fractions may be due to a splicing variant of MSRA, which is localized in the mitochondria, by contamination of the microsomal fraction. The MSRA splicing variant contains a mitochondrial transport signal in the N-terminus and has the ability to catalyze the reduction reaction of *S*-methionine sulfoxide, an endogenous substrate (Vougier et al., 2003; Kim and Gladyshev, 2006). It has been reported that the cytosolic form of MSRA is more abundant than the mitochondrial form in the liver and kidney (Kim and Gladyshev, 2006). Our result showing higher sulindac reductase activity in the cytosolic fraction than in the microsomal fraction is consistent with the localization of MSRA.

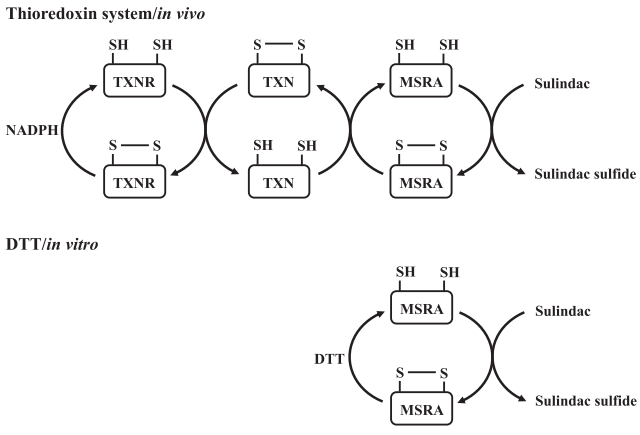

Fig. 7. Possible catalytic mechanisms of MSRA in vivo and in vitro.

MSRA catalyzes the reduction of *S*-methionine sulfoxide, whereas members of the MSRB family (MSRB1, B2, B3), another family of MSRs, catalyze the reduction of *R*-methionine sulfoxide (Moskovitz et al., 2000, 2002; Bar-Noy and Moskovitz, 2002). MSRA has low amino acid homology with MSRB family members (14.6–22.3%) since MSRA is derived from a different evolutionary lineage than MSRB family members (Tamura, 2014). Etienne et al. (2003) showed that MsrA in *E. coli* selectively reduced the *S*-epimer of sulindac. Although this study did not examine whether either the *S*-epimer or *R*-epimer of sulindac is reduced, it is considered that human MSRA may also selectively reduce the *S*-epimer. Further study is needed to confirm the possibility.

In this study, it was revealed that cytosolic factor(s) efficiently enhance MSRA activity (Fig. 4B). Although MSRA-catalyzing sulindac reductase activity was enhanced by the addition of recombinant TXN (Fig. 4C), the estimation method using the TXN protein level and the method using TXN-specific activity in tissue cytosols and recombinant TXN indicated that TXN was not a principal factor in increasing MSRA enzyme activity (Fig. 4, D and E). Since DTT activates MSRA but not TXN by reducing the disulfide bonds between cysteine residues in MSRA in vitro (Fig. 7), TXN may activate MSRA in an additive manner in the presence of DTT. Therefore, the principal factor(s) in increasing MSRA activity might allosterically activate MSRA rather than acting on the cysteine residue in the active center. It is known that MSRA plays a role in maintaining protein functions by catalyzing the reduction of oxidized methionine residues in various proteins (Moskovitz et al., 1996). The oxidation of methionine residues in proteins caused by oxidative stress is relevant to the onset and progression of Alzheimer's and Parkinson's diseases (Yan et al., 2013). In addition, Reiterer et al. (2019) reported that MSRA dysfunction is associated with Alzheimer's disease and Parkinson's disease. Thus, since the factor(s) that increase MSRA activity might be potential therapeutic targets for these diseases, such studies are warranted.

It was originally reported that sulindac is reduced by the gut microbiota (Strong et al., 1985, 1987). To calculate the in vivo contribution of tissue MsrA to sulindac activation, the mouse gut microbiota was depleted by administration of antibiotics. Contrary to our expectation, the preadministration of antibiotics decreased the AUC and shortened the T_{max} of sulindac (Fig. 6, E and F, Table 4). It has been reported that antibiotics such as erythromycin and cephalosporin accelerate gastric emptying (Urbain et al., 1990; Kuo et al., 1998). Thus, the antibiotics used in this study, i.e., ampicillin, metronidazole, and gentamicin, also likely accelerated gastric emptying, causing the rapid transport of sulindac to the small intestine and the shortening of T_{max} of both sulindac and sulindac sulfide. As a result, the absorption rate of sulindac in the upper small intestine may have been reduced. Such phenomena have been reported for other drugs, such as atazanavir, ritonavir, and acetaminophen (Dossou-Yovo et al., 2014; Malfatti et al., 2020). Because antibiotic treatment affected sulindac absorption, the contribution of tissue MsrA to sulindac activation was estimated by the ratio of the AUC values of sulindac sulfide and sulindac. The contribution of MsrA in

mouse tissues to sulindac activation in the body was calculated to be 69% (Fig. 6, E and F, Table 4). Thus, MsrA in tissues rather than the gut microbiota is a main contributor to sulindac activation. The association of genetic polymorphisms of human MSRA with obesity, diabetes, and coronary artery disease (Hotta et al., 2010; Yeung et al., 2011; Gu et al., 2013; Ma et al., 2019) has been reported, although studies assessing the impact of individual mutations on the enzymatic activity and/or expression of MSRA remain to be performed. It is worth investigating whether sulindac has excess or insufficient pharmacological effects in patients with MSRA variants.

In conclusion, this study clarified that human MSRA in tissues rather than the gut microbiota mainly contributes to sulindac activation. This is the first report demonstrating the role of human MSRA in drug metabolism. Further characterization of MSRA as a drug-metabolizing reductase would be beneficial for pharmacokinetic studies in drug development.

Authorship Contributions

Participated in research design: Hirosawa, Fukami, Nakano, Nakajima.

Conducted experiments: Hirosawa, Nagaoka.

Contributed new reagents or analytic tools: Hirosawa.

Performed data analysis: Hirosawa.

Wrote or contributed to the writing of the manuscript: Hirosawa, Fukami, Nakajima.

References

Abet V, Filace F, Recio J, Alvarez-Builla J, and Burgos C (2017) Prodrug approach: An overview of recent cases. *Eur J Med Chem* **127**:810–827.

Ahmed NK, Felsted RL, and Bachur NR (1979) Comparison and characterization of mammalian xenobiotic ketone reductases. *J Pharmacol Exp Ther* **209**:12–19.

Bar-Noy S and Moskovitz J (2002) Mouse methionine sulfoxide reductase B: effect of selenocysteine incorporation on its activity and expression of the seleno-containing enzyme in bacterial and mammalian cells. *Biochem Biophys Res Commun* **297**:956–961.

Berg AK, Mandrekar SJ, Ziegler KLA, Carlson EC, Szabo E, Ames MM, Boring D, Limburg PJ, and Reid JM; Cancer Prevention Network (2013) Population pharmacokinetic model for cancer chemoprevention with sulindac in healthy subjects. *J Clin Pharmacol* **53**: 403–412.

Brogden RN, Heel RC, Speight TM, and Avery GS (1978) Sulindac: a review of its pharmacological properties and therapeutic efficacy in rheumatic diseases. *Drugs* **16**:97–114.

Brunell D, Sagher D, Kesaraju S, Brot N, and Weissbach H (2011) Studies on the metabolism and biological activity of the epimers of sulindac. *Drug Metab Dispos* **39**:1014–1021.

Cerny MA (2016) Prevalence of Non-Cytochrome P450-Mediated Metabolism in Food and Drug Administration-Approved Oral and Intravenous Drugs: 2006-2015. *Drug Metab Dispos* **44**:1246–1252.

Dossou-Yovo F, Mamadou G, Soudy ID, Limas-Nzouzi N, Miantezila J, Desjeux JF, and Eto B (2014) Metronidazole or Cotrimoxazole therapy is associated with a decrease in intestinal bioavailability of common antiretroviral drugs. *PLoS One* **9**:e89943.

Duggan DE, Hooke KF, Risley EA, Shen TY, and Arman CG (1977a) Identification of the biologically active form of sulindac. *J Pharmacol Exp Ther* **201**:8–13.

Duggan DE, Hare LE, Ditzler CA, Lei BW, and Kwan KC (1977b) The disposition of sulindac. *Clin Pharmacol Ther* **21**:326–335.

Etienne F, Resnick L, Sagher D, Brot N, and Weissbach H (2003) Reduction of Sulindac to its active metabolite, sulindac sulfide: assay and role of the methionine sulfoxide reductase system. *Biochem Biophys Res Commun* **312**:1005–1010.

Fukami T, Kariya M, Kurokawa T, Iida A, and Nakajima M (2015) Comparison of substrate specificity among human arylacetamide deacetylase and carboxylesterases. *Eur J Pharm Sci* **78**:47–53.

Gingell R, Bridges JW, and Williams RT (1971) The role of the gut flora in the metabolism of prontosil and neoprontosil in the rat. *Xenobiotica* **1**:143–156.

Gu H, Chen W, Yin J, Chen S, Zhang J, and Gong J (2013) Methionine sulfoxide reductase A rs10903323 G/A polymorphism is associated with increased risk of coronary artery disease in a Chinese population. *Clin Biochem* **46**:1668–1672.

Hisamuddin IM and Yang VW (2007) Genetic polymorphisms of human flavin-containing monooxygenase 3: implications for drug metabolism and clinical perspectives. *Pharmacogenomics* **8**:635–643.

Holmgren A (1989) Thioredoxin and glutaredoxin systems. *J Biol Chem* **264**:13963–13966.

Hotta K, Nakamura M, Nakamura T, Matsuo T, Nakata Y, Kamohara S, Miyatake N, Kotani K, Komatsu R, Itoh N, et al. (2010) Polymorphisms in NRXN3, TFAP2B, MSRA, LYPLAL1, FTO and MC4R and their effect on visceral fat area in the Japanese population. *J Hum Genet* **55**:738–742.

Kim HY and Gladyshev VN (2006) Alternative first exon splicing regulates subcellular distribution of methionine sulfoxide reductases. *BMC Mol Biol* **7**:11.

Kitamura S, Sugihara K, Hosokawa R, Akagawa Y, Ohta S, Ohashi KNK (2001) Extremely high drug-reductase activity based on aldehyde oxidase in monkey liver. *Biol Pharm Bull* **24**:856–859.

Klotz U (1985) Clinical pharmacokinetics of sulphasalazine, its metabolites and other prodrugs of 5-aminosalicylic acid. *Clin Pharmacokinet* **10**:285–302.

Kobayashi M, Higuchi S, Mizuno K, Tsuneyama K, Fukami T, Nakajima M, and Yokoi T (2010) Interleukin-17 is involved in α-naphthylisothiocyanate-induced liver injury in mice. *Toxicology* **275**:50–57.

Konishi K, Fukami T, Gotoh S, and Nakajima M (2017) Identification of enzymes responsible for nitrazepam metabolism and toxicity in human. *Biochem Pharmacol* **140**:150–160.

Kuo WH, Wadwa KS, and Ferris CD (1998) Cephalosporin antibiotics accelerate gastric emptying in mice. *Dig Dis Sci* **43**:1690–1694.

Kwak GH, Choi SH, Kim JR, and Kim HY (2009) Inhibition of methionine sulfoxide reduction by dimethyl sulfoxide. *BMB Rep* **42**:580–585.

Lloyd P, Flesch G, and Dieterle W (1994) Clinical pharmacology and pharmacokinetics of oxcarbazepine. *Epilepsia* **35** (Suppl 3):S10–S13.

Lowther WT, Brot N, Weissbach H, Honek JF, and Matthews BW (2000) Thiol-disulfide exchange is involved in the catalytic mechanism of peptide methionine sulfoxide reductase. *Proc Natl Acad Sci USA* **97**:6463–6468.

Lundström J and Holmgren A (1990) Protein disulfide-isomerase is a substrate for thioredoxin reductase and has thioredoxin-like activity. *J Biol Chem* **265**:9114–9120.

Ma Z, Wang Y, Xu C, Ai F, Huang L, Wang J, Peng J, Zhou Y, Yin M, Zhang S, et al. (2019) Obesity-Related Genetic Variants and Hyperuricemia Risk in Chinese Men. *Front Endocrinol (Lausanne)* **10**:230.

Malfatti MA, Kuhn EA, Murugesh DK, Mendez ME, Hum N, Thissen JB, Jaing CJ, and Loots GG (2020) Manipulation of the Gut Microbiome Alters Acetaminophen Biodisposition in Mice. *Sci Rep* **10**:4571.

Moskovitz J, Poston JM, Berlett BS, Nosworthy NJ, Szczepanowski R, and Stadtman ER (2000) Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. *J Biol Chem* **275**:14167–14172.

Moskovitz J, Singh VK, Requena J, Wilkinson BJ, Jayaswal RK, and Stadtman ER (2002) Purification and characterization of methionine sulfoxide reductases from mouse and Staphylococcus aureus and their substrate stereospecificity. *Biochem Biophys Res Commun* **290**:62–65.

Moskovitz J, Weissbach H, and Brot N (1996) Cloning the expression of a mammalian gene involved in the reduction of methionine sulfoxide residues in proteins. *Proc Natl Acad Sci USA* **93**:2095–2099.

Penning TM (2015) The aldo-keto reductases (AKRs): Overview. *Chem Biol Interact* **234**: 236–246.

Radi ZA and Khan NK (2006) Effects of cyclooxygenase inhibition on the gastrointestinal tract. *Exp Toxicol Pathol* **58**:163–173.

Reiterer M, Schmidt-Kastner R, and Milton SL (2019) Methionine sulfoxide reductase (Msr) dysfunction in human brain disease. *Free Radic Res* **53**:1144–1154.

Sakai Y, Fukami T, Nagaoka M, Hirosawa K, Ichida H, Sato R, Suzuki K, Nakano M, and Nakajima M (2021) Arylacetamide deacetylase as a determinant of the hydrolysis and activation of abiraterone acetate in mice and humans. *Life Sci* **284**:119896.

Strong HA, Renwick AG, George CF, Liu YF, and Hill MJ (1987) The reduction of sulphinpyrazone and sulindac by intestinal bacteria. *Xenobiotica* **17**:685–696.

Strong HA, Warner NJ, Renwick AG, and George CF (1985) Sulindac metabolism: the importance of an intact colon. *Clin Pharmacol Ther* **38**:387–393.

Sun J, Xu J, Ling Y, Wang F, Gong T, Yang C, Ye S, Ye K, Wei D, Song Z, et al. (2019) Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl Psychiatry* **9**:189.

Sung JW, Yun HY, Park S, Kim YJ, Yee J, Lee KE, Song B, Chung JE, and Gwak HS (2020) Population pharmacokinetics of sulindac and genetic polymorphisms of FMO3 and AOX1 in women with preterm labor. *Pharm Res* **37**:44.

Takeno S and Sakai T (1991) Involvement of the intestinal microflora in nitrazepam-induced teratogenicity in rats and its relationship to nitroreduction. *Teratology* **44**:209–214.

Tamura T (2014) MSR-A and -B work on S- and R-isomers of Met-sulfoxide. *VITAMINS* **88**:102–105.

Tanaka Y (1993) Stereoselective metabolism studies on loxoprofen, a 2-arylpropionic acid nonsteroidal anti-inflammatory drug, and its role for the development as a prodrug. *Drug Metab Pharmacokinet* **8**:521–536.

Tang YJ, Hu K, Huang WH, Wang CZ, Liu Z, Chen Y, Ouyang DS, Tan ZR, Zhou HH, and Yuan CS (2017) Effects of FMO3 polymorphisms on pharmacokinetics of sulindac in Chinese healthy male volunteers. *BioMed Res Int* **2017**:4189678.

Tsuchiya Y, Nakajima M, Kyo S, Kanaya T, Inoue M, and Yokoi T (2004) Human CYP1B1 is regulated by estradiol via estrogen receptor. *Cancer Res* **64**:3119–3125.

Urbain JLC, Vantrappen G, Janssens J, Van Cutsem E, Peeters T, and De Roo M (1990) Intravenous erythromycin dramatically accelerates gastric emptying in gastroparesis diabeticorum and normals and abolishes the emptying discrimination between solids and liquids. *J Nucl Med* **31**:1490–1493.

Vougier S, Mary J, and Friguet B (2003) Subcellular localization of methionine sulphoxide reductase A (MsrA): evidence for mitochondrial and cytosolic isoforms in rat liver cells. *Biochem J* **373**:531–537.

Wolpert MK, Althaus JR, and Johns DG (1973) Nitroreductase activity of mammalian liver aldehyde oxidase. *J Pharmacol Exp Ther* **185**:202–213.

Yan MH, Wang X, and Zhu X (2013) Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radic Biol Med* **62**:90–101.

Yeung E, Qi L, Hu FB, and Zhang C (2011) Novel abdominal adiposity genes and the risk of type 2 diabetes: findings from two prospective cohorts. *Int J Mol Epidemiol Genet* **2**:138–144.

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Methionine sulfoxide reductase A in human and mouse tissues is responsible for sulindac activation, making a larger contribution than the gut microbiota

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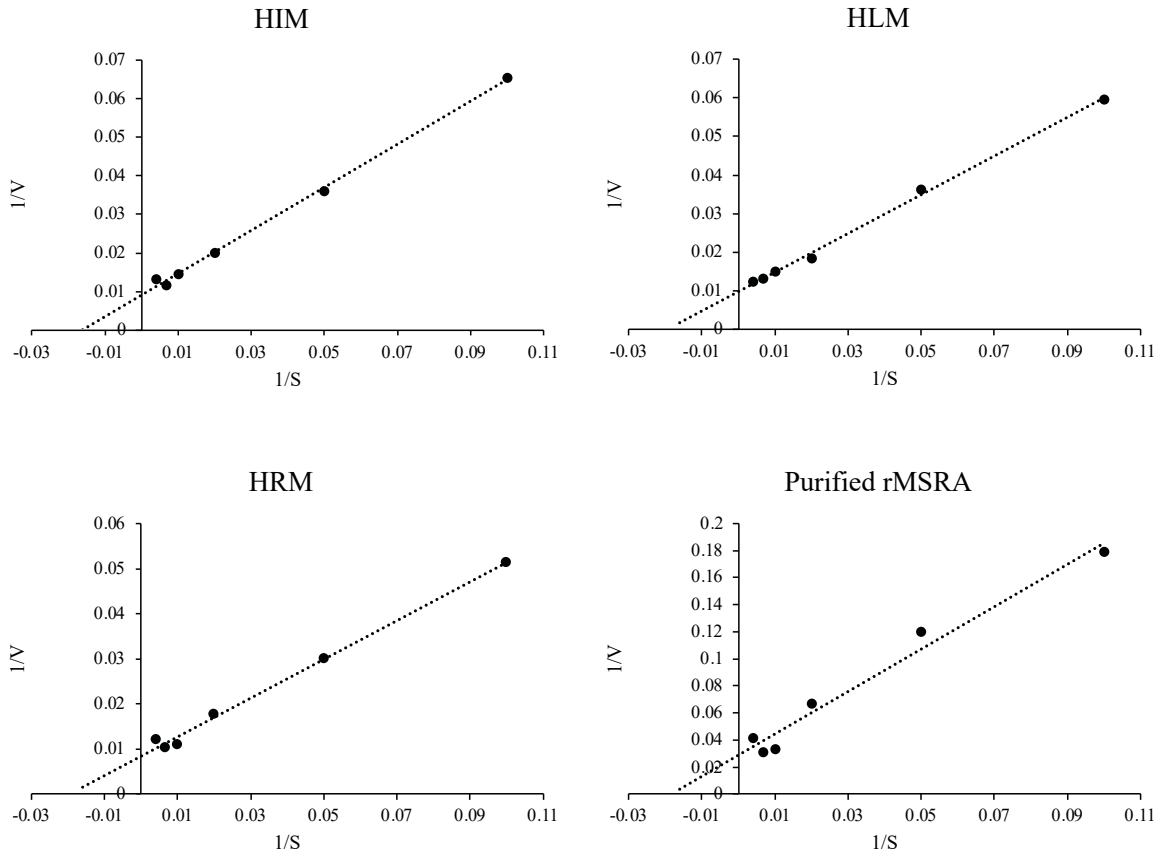
Supplementary Fig. 1S



Supplementary Fig. 1S. Lineweaver-Burk plots of sulindac reductase activities by HIM, HLM, HRM, and purified recombinant MSRA.