Biochemical Pharmacology 81 (2011) 1317–1323

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Sulindac sulfide induces autophagic death in gastric epithelial cells via Survivin down-regulation: A mechanism of NSAIDs-induced gastric injury

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ARTICLE INFO

Article history:
Received 27 January 2011
Accepted 22 March 2011
Available online 30 March 2011

Keywords:
NSAIDs
Survivin
Autophagy
Cell death
Gastric mucosal injury
Sulindac sulfide

ABSTRACT

Sulindac sulfide, a nonsteroidal anti-inflammatory drug (NSAID), has anti-tumorigenic and anti-inflammatory activities, but causes gastric mucosal damage. NSAIDs cause gastric injury in part by down-regulation of Survivin, an apoptosis inhibitor, resulting in apoptosis induction. Autophagy is a process that promotes cellular health by destroying unwanted cellular materials. Excessive autophagy induction could lead to a non-apoptotic cell death (autophagic cell death). The present study showed that sulindac sulfide at a physiological concentration also induces autophagic death in human gastric epithelial AGS and rat gastric epithelial RGM-1 cells, and that Survivin down-regulation is a mechanism involved: Sulindac sulfide treatment increased LC3b-II and APG7 levels and cytosolic vacuole formation, indications of autophagy induction, in AGS and RGM-1 cells. Sulindac sulfide treatment induced AGS and RGM-1 cell death, which was significantly reduced by pretreatment with the autophagy inhibitors 3-methyladenine and chloroquine, indicating that sulindac sulfide induced autophagic cell death. Stable overexpression of Survivin in RGM-1 cells did not inhibit the induction of LC3b-II levels or vacuole formation by sulindac sulfide, but significantly reduced the resulting cell death, suggesting that Survivin may inhibit autophagic cell death downstream of LC3b-II induction and vacuole formation. Indeed, siRNA depletion of LC3b in AGS cells inhibited the down-regulation of Survivin levels and the induction of cell death by sulindac sulfide, confirming that down-regulation of Survivin occurs in the autophagy pathway downstream of LC3b-II induction by sulindac sulfide. Induction of Survivin-dependent autophagic cell death is a novel mechanism by which sulindac sulfide induces gastric mucosal injury.
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1. Introduction

Sulindac sulfide is a nonsteroidal anti-inflammatory drug (NSAID). It has known pro-apoptotic, anti-tumorigenic, and anti-inflammatory activities, and is used to treat familial adenomatous polyposis. The physiological concentration of sulindac sulfide, based on the blood levels in patients treated with the drug, is around 0.01 mM [1]. In a microarray analysis, sulindac sulfide given at this concentration to colon cancer cells in culture is effective in modulating several genes that are linked to regulation of apoptosis and tumorigenesis [2]. Because of its physiological relevance and its effectiveness in illiciting relevant biochemical responses in cultured cells, we also utilized this sulindac sulfide concentration for this study.

Most of what is known about sulindac sulfide's mechanisms of action is described in the context of cancer. However, similar to other NSAIDs, sulindac sulfide frequently causes severe side-effects in patients such as gastric mucosal erosions and ulcers. Thus it is also essential to understand the underlying mechanisms of how sulindac sulfide induces gastric mucosal injury. The damaging effects of sulindac sulfide in the gastric mucosa can be attributed in part to its pro-apoptosis activity. We previously showed that one mechanism by which NSAIDs induce apoptosis is by reducing the levels of Survivin, a known apoptosis inhibitor, in rat gastric epithelial RGM-1 cells in culture, and *in vivo* in rat gastric mucosa [3]. Overexpression of Survivin effectively inhibited NSAIDs-induced apoptosis in cultured RGM-1 cells [3,4]. Surprisingly, overexpression of Survivin also resulted in a significant reduction of all modes of cell deaths induced by NSAIDs, of which apoptotic cell death constituted only a minor percentage [3,4]. The non-apoptotic modes of cell death induced by NSAIDs were not identified at the time. A general cytoxicity assay that measures lactate dehydrogenase (LDH) release from dead cells was utilized to determine the total amounts of apoptotic plus non-apoptotic cell deaths resulting from NSAIDs treatments, and was also utilized in this study.

Macroautophagy, or autophagy, is an evolutionarily conserved process that maintains cellular health and ensures cell survival by digesting and removing damaged/unwanted cellular materials such as organelles, proteins and macromolecules [5,6]. It is

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0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2011.03.019

inducible in response to cellular stresses, including nutrient deprivation and oxidative stress. When induced, it functions to break down protein aggregates into amino acids that are then recycled by the cell for its metabolic and survival needs [6]. However, excessive autophagy induction will trigger a non-apoptotic cell death, also termed "type II programmed cell death". Autophagy is initiated by the formation of double-membrane vacuoles, autophagosomes, that quarantine the unwanted cytosolic materials and subsequently fuses with the lysosomes to degrade its contents. Autophagosome formation requires cleavage of microtubule-associated protein light chain 3b (LC3b) by Apg 4 to form cytosolic LC3b-I [7]. LC3b-I undergoes conjugation to a lipid moiety, by the Apg 7 and Apg 3 enzymes, to generate LC3b-II, which incorporates into the autophagosome membrane with aid from a Apg12–Apg 5–Apg16 protein complex [8,9]. Together, the entire complex is also responsible for the elongation and curvature of the vacuole to generate a mature autophagosome [10,11]. Detections of LC3b-II by western blotting, and of the punctate distribution of LC3b-II in cytosolic vacuole membranes by immunofluorescence, are two of the most commonly utilized and widely accepted assays for monitoring the autophagic flux. Changes in levels of other proteins involved in autophagy, such as the Apg proteins, are other methods for detection of autophagy activation. In this study, one or combinations of these detection methods were utilized in each experiment.

Recently, Survivin is implicated in the inhibition of prostate cancer cell death that results from autophagy. The CCL2 chemokine induces PI3-K/AKT-dependent up-regulation of Survivin, which inhibits autophagic death in PC3 cells [12]. Also, ectopic over-expression of Survivin inhibits apoptosis that is dependent on autophagy induction in PC3 cells treated with a Survivin suppressing drug, YM155 [13].

The possibility arises that induction of autophagic cell death is a non-apoptotic mechanism, also triggered by the down-regulation of Survivin, by which sulindac sulfide destroys gastric epithelial cells. In this study, we examined the possibility that sulindac sulfide induces autophagic death in gastric epithelial AGS and RGM-1 cells, and that Survivin down-regulation is a mechanism involved.

2. Materials and methods

2.1. Cell lines and treatments

Human gastric carcinoma AGS cells (ATCC, Manassas, VA) and normal rat gastric mucosal epithelial RGM-1 cells [14] were cultured as described previously [15]. We chose to utilize AGS cells in this study because normal human gastric epithelial cells are difficult to establish in culture, and are not readily available. AGS cells are frequently utilized as a cell culture model for human gastric epithelial cells. We also performed our studies in RGM-1 cells in order to confirm the results in a normal gastric epithelial cell model.

Cells were grown in 6-well culture plates until they were about 70% confluent. To avoid spontaneous induction of autophagy in completely serum free media, cells were then incubated overnight in reduced-serum media (0.5% FBS). Incubation of cells in reduced serum media prior to NSAIDs treatments was necessary since high serum concentrations interfere with the cytotoxic effects of NSAIDs. Then cells were treated with: (1) vehicle (dimethylsulfoxide), 0.01 mM sulindac sulfide, 0.05 mM chloroquine for 0, 12, 24 and 36 h to examine the timing of LC3b-II induction; (2) vehicle, 0.01 mM sulindac sulfide, and 0.05 mM chloroquine for 24 h to detect LC3b-II incorporation in cytosolic vacuoles; (3) vehicle, 0.01 mM sulindac sulfide, 0.05 mM chloroquine, 10 mM 3-methyladenine, and combinations of sulindac sulfide and 3-methyladenine or chloroquine for 0, 12, 24 and 36 h to examine the timing of induction and inhibition of autophagic death; and (4) vehicle, 0.01 mM sulindac sulfide for 24 h to examine apoptosis induction. For the combination treatments, cells were pretreated with 3-methyladenine or chloroquine for 20 min before sulindac sulfide was added. All chemicals described above were purchased from Sigma–Aldrich, St. Louis, MO.

2.2. Microscopic detection of LC3b translocation and vacuole formation

Autophagosome formation was detected utilizing the Premo Autophagy Sensors (LC3B-FP) BacMam 2.0 kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The Premo Autophagy Sensors (LC3B-FP) BacMam 2.0 kit allows transduction of cultured mammalian cells with an LC3b-fluorescent protein chimera (LC3B-FP), and a negative control fluorescent LC3b protein containing a mutation that renders the protein unable to be processed to form LC3b-II-FP (LC3B(G120A)-FP). Transduction occurs via an insect Baculovirus vector containing a mammalian promoter. Transduced cells were cultured and treated in the same way as described above. Incorporation of LC3b-II-FP in cytsolic vacuoles was visualized with a Nikon Optiphot epifluorescence microscope (Nikon, Inc., Melville, NY) with Omega filter fluorescein isothiocyanate/Texas red, at 100× magnification. Induction of vacuolization in cells was visualized by phase contrast microscopy at 100× magnification.

2.3. Fluorescence flow cytometric quantitation of APG7 levels

10^4 AGS and RGM-1 cells per well were seeded and grown in reduced-serum medium overnight. Then triplicate wells were treated with vehicle and 0.01 mM sulindac sulfide for 24 h. Cells were then fixed in 4% paraformaldehyde (Sigma, St. Louis, MO), and permiabilized with methanol (Fisher Scientific, Pittsburgh, PA). Then cells were incubated with APG7 rabbit polyclonal antibody (ProSci, Inc., Poway, CA) for 2 h, then with FITC conjugated goat-anti-rabbit antibody for (Sigma, St. Louis, MO) 1 h. Fluorescence intensity data was obtained using NOVAStar (BMG LABTECH, Durham, NC) as described previously [16].

2.4. Cell death and apoptosis assays

Percent cell death was determined utilizing the Cytoscan LDH Assay Kit (Bioworld, Dublin, OH), according to the manufacturer's instructions, and as described previously [4]. Percent apoptosis was determined utilizing the annexin-V binding assay as described previously [4].

2.5. Western blot analysis

Cell lysates were prepared as described previously [15]. 150 μg protein per sample was separated by 12% SDS–PAGE and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Piscataway, NJ). The membranes were blocked in skim milk and incubated with rabbit-polyclonal anti- LC3b antibodies (Novus Biologicals, Littleton, CO) at 4 °C overnight. The membrane was then washed and incubated for 1 h with peroxidase conjugated goat-anti-rabbit secondary antibodies (Sigma, St. Louis, MO). The same membrane was then stripped with Re-blot Mild solution (Millipore, Temecula, CA) according to the manufacturer's instructions, on the Lab Line rotator (Fisher Scientific, Chino, CA) to ensure uniform stripping throughout the entire membrane, and then incubated with mouse monoclonal anti-β-actin antibodies (Sigma, St. Louis, MO) for 1 h, washed, and incubated for 1 h with peroxidase conjugated goat-anti-mouse secondary antibodies

(Sigma, St. Louis, MO). The same membrane was stripped for the final time and incubated overnight with anti-rabbit polyclonal Survivin antibodies (Novus Biologiocals, Littleton, CO), washed, and incubated for 1 h with peroxidase conjugated goat-anti-rabbit secondary antibodies (Sigma, St. Louis, MO).

2.6. LC3 siRNA transfections in AGS cells

A commercially available pool of 3 target-specific siRNAs designed to silence human LC3b (Santa Cruz Biotechnologies, Santa Cruz CA) was utilized. 10 nM of siRNAs was transfected into AGS cells at 70% confluence using RNAiMax transfection reagent (Invitrogen, Carlsbad CA), following the manufacturer's instructions. Mock transfection utilizing only the transfection reagent but no siRNA, and transfection of 10 nM of a commercially available siRNA that does not silence mammalian mRNAs (Santa Cruz Biotechnologies, Santa Cruz CA) were included as controls. The non-silencing control siRNA is conjugated to a green fluorescent dye for monitoring transfection efficiency with fluorescence microscopy. The percentage of fluorescent cells per 500 total cells was counted to determine the transfection efficiency. 24 h post transfection, cells were incubated in reduced serum media overnight. Then cells were treated with vehicle and sulindac sulfide (0.01 mM) for 24 h. At this time cell lysates were collected for western blot analysis of LC3b-II, Survivin, and beta actin levels. LDH and annexin-V assays were also performed at this time as described previously [4].

2.7. Stable Survivin overexpressing RGM-1 cell lines and treatments

Establishment and culture of a RGM-1 cell line that stably overexpress Survivin, and the accompanying control cell line that contains stably integrated empty parental vector, were described previously [4]. Each cell line was grown in 6 well culture plates until they were about 70% confluent. Then they were incubated overnight in reduced serum media, and subsequently treated with vehicle, 0.01 mM sulindac sulfide (Sigma, St. Louis, MO) alone or with 0.05 mM chloroquine pretreatment (Sigma, St. Louis, MO). 24 h later, percent cell death and apoptosis were determined as previously described [4], LC3b-II induction was determined by western blot analysis.

2.8. Statistical analysis

All data subjected to statistical analysis were performed in triplicates. Student's *t* test was used to compare data between two conditions. p-Value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Sulindac sulfide treatment at physiological concentration induced autophagy in gastric epithelial cells

Autophagy induction was monitored in gastric epithelial cells by three different methods: (1) western blot detection of LC3b-II, (2) fluorescence microscope detection of LC3b-II-FP incorporation in cytosolic vacuoles, and (3) flow cytometric detection of APG7 up-regulation.

We first examined whether sulindac sulfide induces LC3b-II in both AGS and RGM-1 cells utilizing western blot analysis in a timecourse study. In both AGS and RGM-1 cells treated with vehicle, LC3b-II levels remained barely detectable for the 36 h duration of the study (Fig. 1A and B). In both AGS and RGM-1 cells, LC3b-II levels increased by 12 h after addition of sulindac sulfide to culture media (Fig. 1A and B). LC3b-II levels peaked at about 24 h after addition of sulindac sulfide, and reduced to basal levels by 36 h after addition of sulindac sulfide (Fig. 1A and B). Chloroquine is an inhibitor of the autophagy process at a late stage. It induces aberrant accumulation of LC3b-II and cytosolic vacuoles, but blocks their subsequent clearance by lysosomes. Therefore, we examined whether LC3b-II levels also cleared by 36 h when autophagy is inhibited by treatment with chloroquine. Unlike sulindac sulfide, in both AGS and RGM-1 cells, chloroquine treatments induced progressive accumulation of LC3b-II throughout the 36 h duration of the study (Fig. 1A and B). Thus sulindac sulfide treatments increased LC3b-II levels, but unlike an autophagy inhibitor, allowed LC3b-II to clear. Since LC3b-II levels peaked at 24 h of sulindac sulfide treatment, we examined autophagy induction by sulindac sulfide treatment at this time point in all subsequent studies.

We next utilized chloroquine to confirm that the transduced LC3B-FP could be effectively processed to LC3b-II-FP and incorporated into cytosolic vacuoles in AGS cells. Cells transduced with LC3B-FP and treated with chloroquine contained large amounts of punctate cytosolic fluorescence signals, indicating that effective LC3B-FP processing and incorporation into vacuoles occurred (Fig. 2B). This was specific to LC3B-FP, since cells transduced with the mutant LC3B(G120A)-FP and treated with chloroquine retained mostly diffuse cytosolic fluorescence, indicating inability of the mutant protein to be processed and incorporated into vacuoles (Fig. 2A).

Since the system worked, we next examined whether sulindac sulfide treatment could also trigger processing and incorporation of transduced LC3B-FP into cytosolic vacuoles in AGS cells. Background levels of punctuate fluorescence were present in a

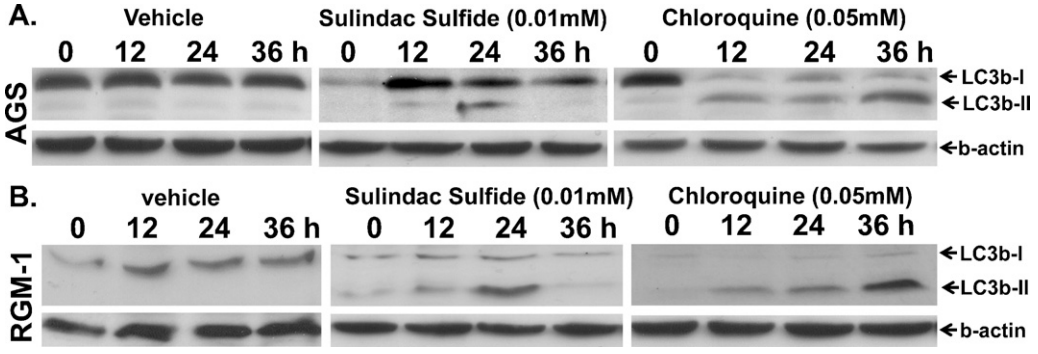

Fig. 1. Timecourse of LC3b-II induction in AGS and RGM-1 cells by physiological sulindac sulfide concentration. Western blot analysis showed that both AGS (A) and RGM-1 (B) cells treated with vehicle contained barely detectable LC3b-II levels throughout the 36 h duration of the study. LC3b-II was visibly increased in both AGS and RGM-1cells at 12 h, peaked at 24 h, and decreased to basal levels by 36 h after sulindac sulfide addition. In contrast, treatment of both AGS and RGM-1 cells with chloroquine, a late stage autophagy inhibitor, induced progressive accumulation of LC3b-II levels throughout the duration of the study. Beta actin blot showed equal total protein loading in all lanes.

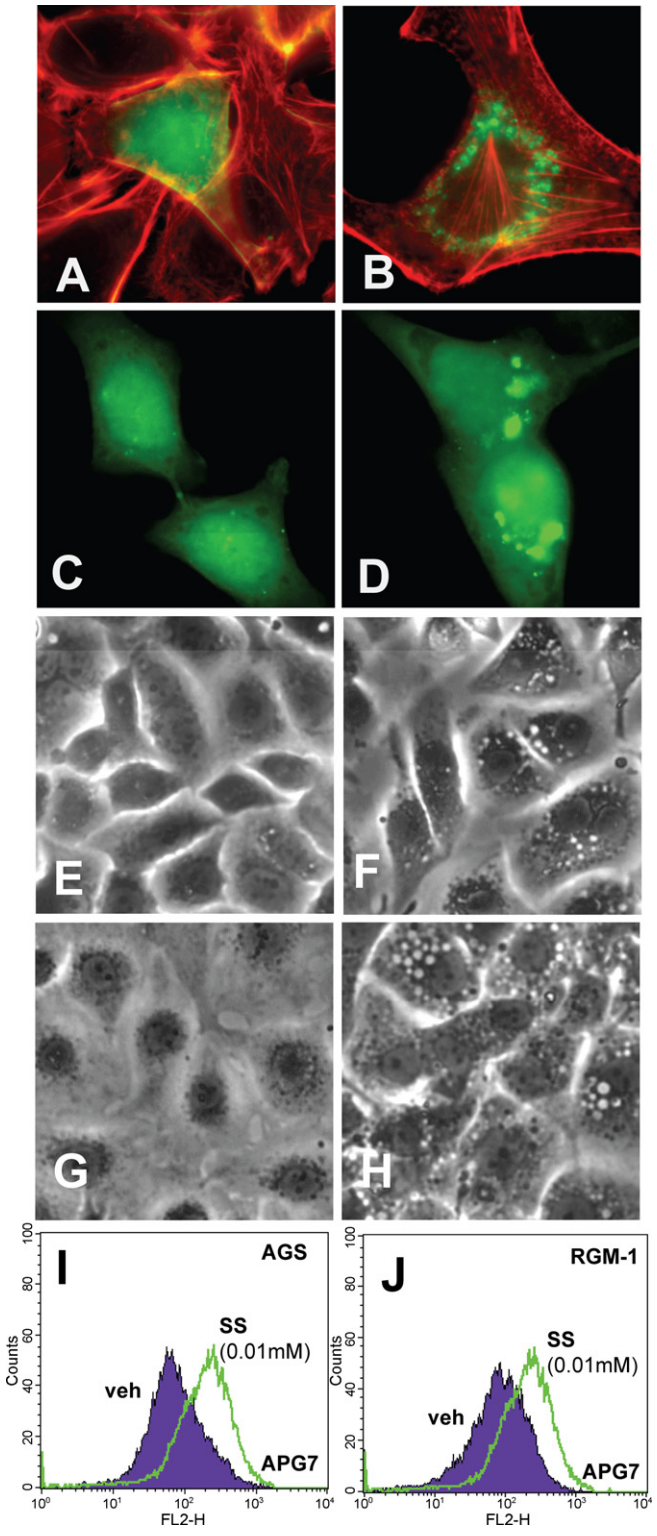

Fig. 2. Induction of cytosolic vacuole formation and APG7 levels in AGS and RGM-1 cells by physiological sulindac sulfide concentration. AGS cells were transduced with LC3B-FP or a mutant LC3B(G120A)-FP that can not be processed to form LC3b-II. Chloroquine (0.05 mM, 24 h) treatment did not induce LC3B(G120A)-FP processing and incorporation into cytosolic vacuoles (A), but induced LC3B-FP processing and incorporation into cytosolic vacuoles (B). Vehicle treatment at 24 h did not induce LC3B-FP processing and incorporation into cytosolic vacuoles (C). LC3b-II incorporation into cytosolic vacuoles occurred in AGS cells at 24 h of treatment with 0.01 mM sulindac sulfide (D). AGS cells treated with vehicle (E) and RGM-1 cells treated with vehicle (G) contained low background amounts of cytosolic vacuoles. AGS cells treated with sulindac sulfide (F) and RGM-1 cells treated with sulindac sulfide (H) contained much greater amounts of cytosolic vacuole. 100× magnification. FACs analysis showed significant APG7 increase resulting from sulindac sulfide treatment in both AGS (I) and RGM-1 (J) cells. Veh, vehicle; ss, sulindac sulfide (0.01 mM).

small number of vehicle treated cells (Fig. 2C). Examination under phase contrast microscope revealed that vehicle treated cells contain only minimal amounts of cytosolic vacuoles (Fig. 2E). Sulindac sulfide treatment dramatically increased the punctate fluorescence signals in the cells, compared to vehicle treatment (Fig. 2D). Examination under phase contrast microscope showed presence of larger amounts of cytosolic vacuoles in higher numbers of cells in the sulindac sulfide treated compared to vehicle treated cell population (Fig. 2F). Thus, sulindac sulfide induced LC3b-II incorporation into cytosolic vacuoles in AGS cells. Results obtained with the transduction assay were shown only in AGS cells because RGM-1 cells were not effectively transduced using this system.

We examined in RGM-1 cells whether sulindac sulfide could induce vacuole formation and up-regulate APG7 levels. Vehicle treated cells contained low background amounts of cytosolic vacuoles (Fig. 2G). Sulindac sulfide treatment dramatically increased the amount of cytosolic vacuoles (Fig. 2H). Fluorescence flow cytometry showed that, compared to vehicle treated RGM-1 cells, sulindac sulfide treated RGM-1 cells contained significantly higher APG7 fluorescence signal (Fig. 2J). Finally, we confirmed, in AGS cells, that sulindac sulfide treatment also significantly increased APG7 fluorescent signal compared to vehicle treatment (Fig. 2I).

Taken together, these results showed that a physiologically relevant concentration of sulindac sulfide effectively induces autophagy in gastric epithelial cells.

3.2. Sulindac sulfide at physiological concentration induced autophagic death in gastric epithelial cells

To examine the relationship between LC3b-II increase and cell death induction by sulindac sulfide, culture media samples were taken from the above timecourse study (shown in Fig. 1) to perform LDH release assay. The minimal serum concentration that was present in our culture media produced only low levels of background LDH readings within the detection range of the assay. Results from all samples were normalized by subtracting these background levels of LDH readings. In both AGS and RGM-1 cells, vehicle treatment resulted in only minimal background levels of cell death through the duration of the study (Fig. 3). At 12 h after addition of sulindac sulfide, low amounts of cell death were induced (Fig. 3). At 24 h after addition of sulindac sulfide, significant amounts of cell death were induced (Fig. 3). At 36 h after addition of sulindac sulfide, even greater proportions of cells were dead (Fig. 3). Thus, marked increases in LC3b-II levels preceded and were associated with significant amounts of cell death in both AGS and RGM-1 cells.

We also confirmed that sulindac sulfide induced autophagic cell death, and not just cell death by other modes, by showing that autophagy inhibitors could significantly reduce sulindac sulfide-induced cell death. We utilized the inhibitors 3-methyladenine and chloroquine at concentrations that are commonly utilized to effectively inhibit autophagy in a variety of different cell types. Treatments of both AGS and RGM-1 cells with 3-methyladenine and chloroquine alone did not induce significant amounts of cell death up to 24 h, and induced minimal amounts of cell death at 36 h of treatment, compared to treatment with vehicle (Fig. 3). Pretreatment of both AGS and RGM-1 cells with 3-methyladenine and chloroquine before addition of sulindac sulfide significantly reduced the amounts of cell death induced by sulindac sulfide (Fig. 3). Cell death inhibition occurred for the 12, 24 and 36 h time points examined in both the AGS and RGM-1 cells (Fig. 3). Taken

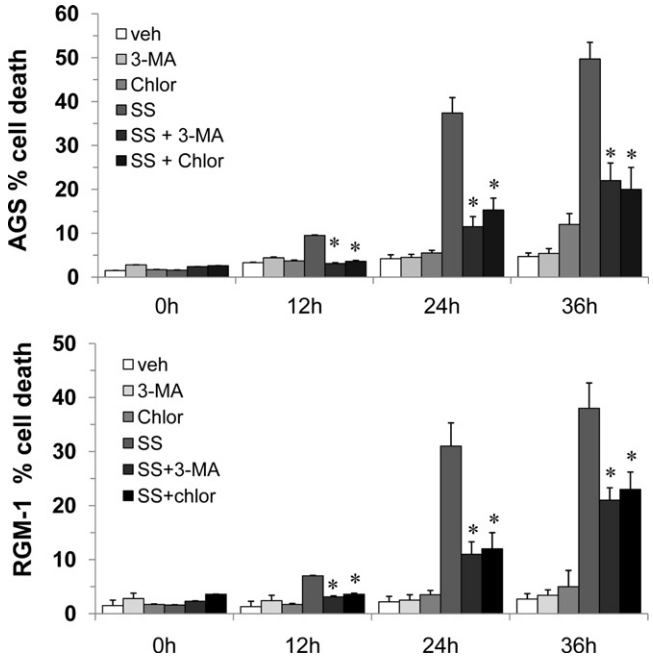

Fig. 3. Timecourse of inhibition of sulindac sulfide-induced cell death by autophagy inhibitors in AGS and RGM-1 cells. LDH release assay showed that treatment of both AGS cells (top graph) and RGM-1 cells (bottom graph) with vehicle, 3-methyladenine and chloroquine did not induce significant percentages of cell death over the entire duration of the study. Treatment with sulindac sulfide induced progressively higher percentages of cell death over the duration of the study. Pretreatments with 3-methyladenine and chloroquine significantly reduced the percentages of sulindac sulfide-induced cell death at 12, 24 and 36 h. All samples are normalized to a positive control, which generated 100% cell death by sonication. Veh, vehicle; ss, sulindac sulfide (0.01 mM), 3-MA, 3-methyladenine (10 mM); Chlor, chloroquine (0.05 mM). *Significant inhibition of percent cell death in the inhibitor pretreated versus sulindac sulfide only treated populations, $p < 0.05$.

together, these results strongly indicated that sulindac sulfide induced autophagic death in gastric epithelial cells.

3.3. Stable Survivin overexpression in RGM-1 cells did not inhibit the increase of LC3b-II levels or vacuole formation, but inhibited the cell death induced by sulindac sulfide at physiological concentration

If Survivin down-regulation is a factor in sulindac sulfide-induced autophagic cell death, then overexpression of Survivin may inhibit induction of autophagy. To explore this possibility, we examined whether ectopic Survivin overexpression would inhibit sulindac sulfide-induced LC3b-II increase and cytosolic vacuole formation. We utilized a stable Survivin overexpressing RGM-1 cell line that was established previously [4]. As control, we utilized an RGM-1 cell line containing stably transfected empty parental vector, as described previously [4]. We previously confirmed that, when compared to the wildtype, untransfected RGM-1 cells, stable transfection of the empty parental vector had no effect on Survivin levels, or on Survivin down-regulation and cell death and apoptosis induction by NSAIDs [4]. We reconfirmed that Survivin levels were dramatically higher in the overexpressing cell line than in the control cell line (Fig. 4A). Sulindac sulfide treatment increased LC3b-II levels in the control cell line, compared to vehicle treatment (Fig. 4B). Sulindac sulfide treatment similarly increased LC3b-II levels in the Survivin overexpressing cell line, compared to vehicle treatment, indicating that Survivin overexpression did not inhibit up-regulation of LC3b-II by sulindac sulfide (Fig. 4B). Vehicle treatment induced minimal background amounts of cytosolic vacuole formation in both control and Survivin overexpressing cell lines (Fig. 4C). Sulindac sulfide treatment induced

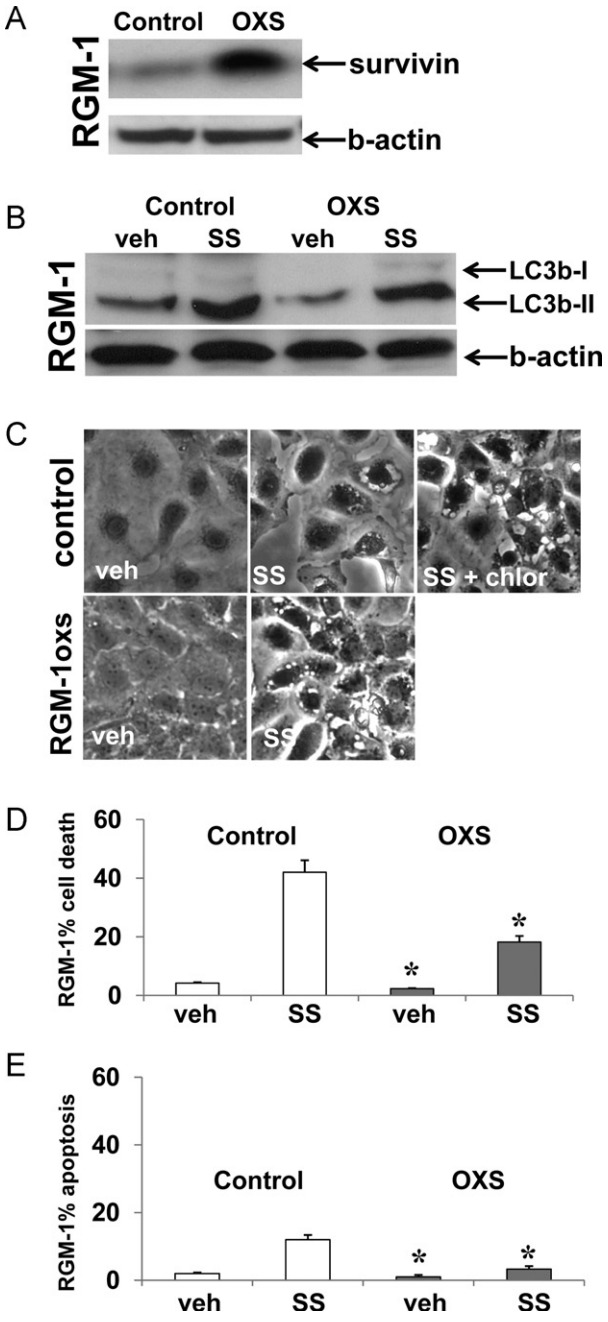

Fig. 4. Stable Survivin overexpression inhibited the cell death, but not the LC3b-II or vacuole induction by sulindac sulfide treatment in RGM-1 cells. (A) Western blot confirming dramatically higher Survivin levels in stable Survivin overexpressing cell line than in control cell line. (B) Western blot showing that LC3b-II induction by sulindac sulfide treatment was not inhibited in the Survivin overexpressing cell line, compared to control cell line. Beta actin blot showed equal total protein loading in all lanes. (C) Phase contrast microscopy showing that greater amounts of vacuole formation was induced by sulindac sulfide in the Survivin overexpressing cell line than in the control cell line. 100× magnification. Both cell death (D) and apoptosis (E) induction by sulindac sulfide were significantly inhibited in the Survivin overexpressing cell line, versus in control cell line. OXS, stable Survivin overexpressing cell line; veh, vehicle; ss, sulindac sulfide (0.01 mM). *Significant inhibition of sulindac sulfide induced cell death and apoptosis in Survivin overexpressing cell line, $p < 0.05$.

greater amounts of vacuole accumulation in the Survivin overexpressing cell line than in the control cell line (Fig. 4C). The amount of vacuole accumulation in the Survivin overexpressing cell line treated with sulindac sulfide was comparable to that in the

control cell line pretreated with chloroquine and then treated with sulindac sulfide (Fig. 4C).

Vehicle treatments induced only minimal amounts of cell death in the control cell line ($3.5 \pm 0.2\%$), and slightly smaller amounts of cell death in Survivin overexpressing cell line ($1.8 \pm 0.13\%$, Fig. 4D). Sulindac sulfide-induced cell death was significantly reduced in the Survivin overexpressing cell line compared to the control cell line ($18.4 \pm 0.8\%$ and $40.6 \pm 2.1\%$, respectively, Fig. 4D). Apoptois accounted for only a minor percentage of cell death induced by sulindac sulfide in both the Survivin overexpressing and control cell lines ($4.5 \pm 0.6\%$ and $13.2 \pm 1.2\%$, respectively, Fig. 4E). Apoptosis also accounted for only a minor percentage of the sulindac sulfide-induced cell death that was inhibited by stable overexpression of Survivin (~9% versus ~22%, Fig. 4E). Taken together, these results indicated that Survivin overexpression did not block sulindac sulfide-induced LC3b-II increase and vacuole formation, but resulted in a significant reduction in the sulindac sulfide-induced non-apoptotic cell death.

3.4. siRNA depletion of LC3b in AGS cells inhibited the Survivin down-regulation, and the autophagic death and apoptosis induction by sulindac sulfide at physiological concentration

To determine whether Survivin down-regulation occurs downstream of LC3b-II induction by sulindac sulfide, we examined whether siRNA depletion of LC3b would inhibit Survivin down-regulation by sulindac sulfide. We performed these studies in human AGS cells since rat LC3b siRNA was not commercially or readily available, and the LC3b siRNA pool we utilized specifically targets human LC3b.

Western blot analysis showed that compared to mock transfection, transfection of the negative control siRNA did not affect LC3b or Survivin protein levels in vehicle treated cells, and did not inhibit the increase of LC3b-II levels or down-regulation of Survivin levels in sulindac sulfide treated cells (Fig. 5A). Transfection of LC3b siRNA significantly reduced LC3-I to undetectable levels in both vehicle and sulindac sulfide treated cells, and thereby blocked the induction of LC3-II protein levels in sulindac sulfide treated cells (Fig. 5A). Transfection of LC3 siRNA increased basal Survivin levels in vehicle treated cells, and blocked the down-regulation of Survivin levels in sulindac sulfide treated cells, compared to mock transfection or control siRNA transfection (Fig. 5A). Control siRNA transfection did not affect the amount of cell death in vehicle or sulindac sulfide-treated cells, compared to mock transfection (Fig. 5B). Transfection of LC3b siRNA slightly decreased the basal levels of cell death in vehicle treated cells, and significantly inhibited the cell death in sulindac sulfide treated cells, compared to mock transfection or control siRNA transfection (Fig. 5B). Apoptosis accounted for only a minor portion of the sulindac sulfide-induced cell death that was inhibited by LC3b depletion (Fig. 5B). Thus, Survivin down-regulation occurs in the autophagy pathway downstream of LC3b-II induction by sulindac sulfide.

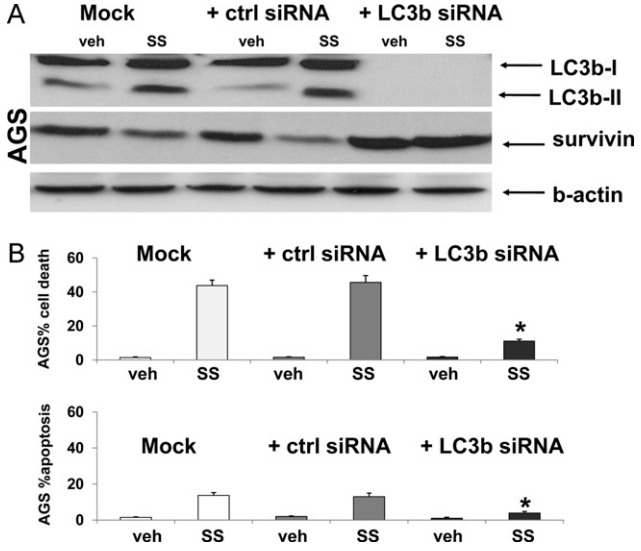

Fig. 5. siRNA depletion of LC3b inhibited the Survivin down-regulation, cell death and apoptosis by sulindac sulfide treatment in AGS cells. (A) Western blot showing that mock transfection and transfection with negative control siRNA did not affect LC3b or Survivin levels, but LC3b siRNA treatment depleted LC3b levels and inhibited Survivin down-regulation by sulindac sulfide treatment. Beta actin blot showed equal total protein loading in all lanes. (B) Mock transfection and transfection with negative control siRNA did not affect general cell death or apoptosis induction by sulindac sulfide treatment. siRNA depletion of LC3b inhibited both general cell death and apoptosis induced by sulindac sulfide treatment. Veh, vehicle; ss, sulindac sulfide (0.01 mM); ctrl siRNA, negative control siRNA. *Significant inhibition of sulindac sulfide induced cell death and apoptosis, compared to negative control siRNA transfection $p < 0.02$.

4. Discussion

In this study, we showed for the first time that a physiologically relevant sulindac sulfide concentration induces autophagy in gastric epithelial cells by demonstrating that sulindac sulfide treatment induces key events in the autophagy process such as LC3b-II and APG7 increases and LC3b-II incorporation into cytosolic vacuoles. We also showed that increases of LC3b-II by sulindac sulfide treatment precedes and is associated with significant cell death. In current literature, there is an abundance of evidence showing induction of autophagy to be a process that promotes cell survival. Inhibition of autophagy often promotes cancer cell death via apoptosis. The existence of autophagic cell death is somewhat a matter for debate. Many view the evidence in the literature to demonstrate that autophagy induction does not lead to cell demise, but cells may die in spite of autophagy induction. Results from this study support the idea that sulindac sulfide induces autophagic death in gastric epithelial cells. Evidence for this was our findings that the autophagy inhibitors 3-methyladenine and chloroquine, as well as LC3b depletion by siRNA, significantly reduced sulindac sulfide-induced non-apoptotic cell death in gastric epithelial cells. If sulindac sulfide triggered cell death alongside autophagy and not as a consequence of it, then we would not expect inhibition of autophagy to inhibit sulindac sulfide-induced cell death.

Sulindac sulfide is known to have anti-tumorigenic activity, and to induce gastric mucosal injury. Results from this study showed that sulindac sulfide, at a physiologically relevant concentration, could induce autophagic death in both gastric carcinoma AGS cells and normal gastric epithelial RGM-1 cells. Thus induction of autophagic cell death may be a contributing mechanism to both the anti-neoplastic and injurious effects of sulindac sulfide.

In this study, we showed that overexpression of Survivin significantly reduced sulindac sulfide-induced cell death in RGM-1 cells. The LDH assay we utilized to measure cell death does not differentiate between apoptotic and non-apoptotic modes of cell death. Survivin is a well-known apoptosis inhibitor. So it was possible that Survivin overexpression inhibited only sulindac sulfide-induced apoptosis but not non-apoptotic modes of cell death. However, our results showed that apoptosis inhibition constituted only a minor portion of the sulindac sulfide-induced cell death that was inhibited by Survivin overexpression. Further, cell death inhibition by Survivin overexpression was accompanied

by accumulation of cytosolic vacuoles, which was reminiscent of chloroquine-inhibition of the autophagic process at a late stage. Indeed, we showed that siRNA depletion of LC3b blocked Survivin down-regulation by sulindac sulfide, indicating that, like chloroquine, Survivin functions downstream of LC3b-II induction and vacuole formation in the autophagy process. Taken together, these results support the idea that Survivin functions downstream of vacuole formation to regulate the sulindac sulfide-induced autophagic cell death pathway.

LC3b is generally known to function only in autophagy. However, our results provided evidence that LC3b may also function to control sulindac sulfide-induced gastric epithelial cell apoptosis, since siRNA depletion of LC3b inhibited sulindac sulfide-induced apoptosis. Our results showed that siRNA depletion of LC3b did not completely inhibit the total cell death induced by sulindac sulfide (Fig. 5). Sulindac sulfide and other NSAIDs are known to induce a number of different types of cell deaths (e.g. apoptosis, autophagic cell death, necrosis, etc.). LC3b is an essential gene for autophagy, and its absence is expected to effectively block autophagic cell death. Therefore, we surmise that the remaining cell death detected in the LC3b siRNA treated cells exposed to sulindac sulfide may not be autophagic cell death. Whether this remaining cell death is due to non-autophagic and non-apoptotic mechanisms, or a combination of apoptotic and non-autophagic mechanisms depends on how effective LC3b is in blocking apoptosis. We were not the first to implicate LC3b in apoptosis regulation. Others have found that LC3 null mice had significantly decreased levels of apoptosis in the lungs after exposure to cigarette smoke, and that LC3 regulates the extrinsic apoptosis pathway in the lung through direct interactions with caveolin-1 and Fas [17]. Results from our study and those of others support the idea that regulation of autophagy is interconnected with regulation of apoptosis, and that the two distinct modes of cell death often share common genetic pathways. Further studies are required to fully understand the molecular mechanisms at the crossroads of autophagy and apoptosis pathways.

Acknowledgement

This work is supported by a Department of Veterans Affairs Merit Review Grant [Grant no. GAST-011-08S] to S.-K. Chiou.

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