Nonsteroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in a variety of cancer cells, including those of colon, prostate, breast and leukemia. In addition, the classical NSAIDs sulindac and aspirin are promising chemopreventive agents against colon cancer. NSAIDs inhibit cyclooxygenases (COX) preventing the formation of prostaglandins, prostacyclin and thromboxane. NSAIDs also exert other biological effects, including generation of reactive oxygen species (ROS) and inhibition of NF-κB-mediated signals. Despite many suggested mechanisms for their anticancer effects, it remains uncertain how they induce cell cycle arrest and apoptosis in cancer cells. Furthermore, there is little information on the selectivity of NSAIDs-mediated anticancer effects, although this is one of the most important issues in cancer therapy. Increased understanding of the biological basis for the anticancer activity of NSAIDs and their selectivity is essential for future therapeutic advances. In this paper, we propose that increased ROS generation is one of the key mechanisms for NSAIDs-mediated anticancer effects on various cancer cells.

Key words: NSAIDs, ROS, DNA damage

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used clinically to control inflammation and pain. They also exert anticancer effects in a variety of cancer cells and epidemiological studies have shown their chemopreventive effect in colon cancer. Many reports confirm that NSAIDs inhibit cell cycle progression and induce apoptosis in cancer cells (Piazza et al., 1997; Subbegowda and Frommel 1998; Grosch et al., 2001). Since all NSAIDs reduce prostaglandin (PG) synthesis by inhibiting cyclooxygenase (COX) activity, this action may be crucial for the mechanism underlying their proapoptotic effects. However, proapoptotic effects of NSAIDs are still observed in a colon cancer cell line, HCT-15, which does not express either COX-1 or COX-2 (Grosch et al., 2001) and in COX-1/-2(-/-) fibroblasts (Zhang et al., 1999). Thus, the proapoptotic effects are likely to be explained by other mechanisms. Although many possibilities have been suggested, the mechanism of NSAIDs action as anticancer or chemopreventive agents remains uncertain. Since NSAIDs have been used clinically for a long time, their efficacy, safety and adverse effects are well recognized, and if they prove to be clinically effective anticancer agents, they would be one of the best agents available.

In this paper, we report that sulindac and its metabolites increase ROS in several human cancer cell lines and propose that oxidative stress may be crucial for NSAIDs-mediated anticancer activity. We also discuss future therapeutic applications of NSAIDs.

Biological effects of NSAIDs

Several molecular explanations underlying the proapoptotic effects of NSAIDs have been advanced (Table 1). As described above, all NSAIDs inhibit COX activity and reduce PG synthesis, but this action appears to be marginal for the proapoptotic effect. However, inhibition of COX activity results in an accumulation of arachidonic acid (AA), since AA is the precursor of PG. Importantly, this accumulated AA can be converted into ceramide by sphyngomyelinase and the subsequent increase of ceramide readily induces apoptosis, suggesting that ceramide may be a crucial mediator for the proapoptotic action of NSAIDs. Furthermore, some NSAIDs inhibit NF-kB-mediated signals (Yamamoto et al., 1999), which strongly induce antiapoptotic molecules, and thus inhibition of NF-kB may also explain how these NSAIDs induce apoptosis. Several investigators, including us, have shown that NSAIDs
strongly induce ROS generation (Giardina and Inan, 1998; Minami et al., 2005). Because various anticancer agents, including cisplatin and taxol, induce oxidative stress, an action indispensable for their anticancer activity (Huang et al., 2003; Park et al., 2004), ROS generation may also be crucial for NSAID-mediated proapoptosis. Furthermore, NSAIDs also modify several intracellular signaling pathways. They activate cyclic GMP-dependent kinase (PKG), through accumulation of cyclic GMP (Soh et al., 2000; Haanen, 2001; Rice et al., 2006). They also activate stress-related kinases JNK and p38, while they inactivate ERK (Rice et al., 2004). These signal modifications may increase susceptibility to apoptotic signals or inhibit survival signals. DNA microarray analysis of more than 8,000 sequences with and without sulindac treatment revealed upregulation of p21WAF1, suggesting an essential role of p21WAF1 for its tumor inhibitory effect (Yang et al., 2001, 2005). Sulindac also inhibits Akt/PKB, which is a major intracellular kinase transducing survival signal (Lee et al., 2005). Interestingly, NSAIDs somehow decrease β-catenin expression levels in colon cancer cells (Rice et al., 2003). Considering that β-catenin is a transcriptional factor, which induces proliferative and survival signals, its inhibition by NSAIDs may explain why NSAIDs are effective in colon cancer cells. Furthermore, sulindac metabolites inhibit estrogen and progesterone receptor expression in breast cancer cells (Lim et al., 2006). These data suggest that colon and breast cancer cells are more sensitive to sulindac and its metabolites than other cancer cells.

**ROS generation in NSAIDs**

Among the many biological effects of NSAIDs described above, we suggest that oxidative stress is a central action for their anticancer effects, since oxidative stress is coupled with many signals inducing apoptosis, such as inhibition of NF-κB and activation of stress-related kinases JNK and p38. Although there have been several conflicting reports regarding ROS generation in NSAIDs-treated cells (Fernandes et al., 2003; Costa et al., 2005), we and others have clearly demonstrated that sulindac and its metabolites can induce ROS generation in several cancer cell lines (Giardina and Inan, 1998; Chung et al., 2003; Minami et al., 2005). We also found that there are considerable differences in the increases of ROS depending upon cell type and time-course. These differences may arise from different anti-oxidant activity in each cell type. In addition, we detected a transient increase in scavenger activity, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) after sulindac treatment in DLD-1 cells (Fig. 1). These observations are consistent with previous reports showing that NSAIDs have antioxidant activity. Since NSAIDs first induce ROS generation, this is one of the biological responses against oxidative stress followed by NSAIDs treatment. We thus propose that the conflicting

---

**Table 1. Biological functions of NSAIDs.**

| (a) | Decrease of prostaglandin (PG) synthesis |
| (b) | Increase of arachidonic acid and ceramide |
| (c) | Inhibition of NF-κB |
| (d) | Increase of cellular cyclic GMP and activation of cyclic GMP-dependent protein kinase (PKG) |
| (e) | Activation of c-jun NH2-terminal kinase (JNK) |
| (f) | Inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) |
| (g) | p21WAF1 induction |
| (h) | Decrease of beta-catenin protein expression |
| (i) | Decrease of estrogen and progesterone receptor expression |

---

**Fig. 1.** Effect of sulindac on SOD, catalase or GPX activity in DLD-1 cells. After incubation with 0.5 mM SUL (closed bars) for the indicated hours (h), SOD (A), catalase (B) or GPX (C) activity was measured. Error bars represent mean ± S.D. *P<0.05; **P<0.01 compared with control.
observations concerning oxidative stress following NSAIDs treatment may be explained by differences in the timing of measurements of ROS or scavenger activity. The molecular mechanism for NSAID-mediated ROS generation remains obscure. Mitochondrial permeability transition (MPT) is a non-selective inner membrane permeabilization that may precede necrotic and apoptotic cell death and may allow abundant leakage of ROS from the mitochondrial electron transport chain (Le Bras et al., 2005). Cyclosporin A (CsA) specifically inhibits this process (Chen et al., 2003), but does not inhibit sulindac-mediated ROS generation. Indeed, MPT is not a consequence of the opening of a pre-formed pore, but results from oxidative damage to pre-existing membrane proteins (Kowaltowski et al., 2001), suggesting that CsA cannot inhibit ROS generation, but acts on the subsequent apoptotic process. In any case, involvement of mitochondrial channels may be marginal in the molecular mechanism(s) by which sulindac induces ROS generation. Alternatively, partial inhibition of mitochondrial respiration is known to enhance electron leakage from the transport chain, leading to increased ROS generation, while a clinically active antileukemia agent, As$_2$O$_3$, inhibits mitochondrial respiratory functions and increases ROS generation (Pelicano et al., 2003). It is thus necessary to investigate whether NSAIDs affect respiratory functions. It is also relevant that NSAIDs induce lipid peroxidation with consequent mitochondrial damage, which may cause electron leakage from the mitochondria. Interestingly, we found that a-tocopherol, but not L-NAC, distinctly inhibited sulindac-induced ROS generation. Considering their different antioxidant functions, i.e., L-NAC increases intracellular content of glutathione and alters redox balance, while a-tocopherol protects membranes from lipid peroxidation (Niki et al., 1989), our data suggest that sulindac may primarily affect membranes and increase lipid peroxidation-mediated ROS. In addition, it is also important to investigate whether NSAIDs can activate NADPH oxidases, since they are one of the major enzymes producing H$_2$O$_2$ and are activated by lipid peroxidation (Li et al., 2003; Ushio-Fukai and Alexander, 2004).

**Comparison of sulindac metabolites**

We used carboxy-H$_2$DCFDA to monitor ROS generation in response to sulindac, its metabolites and other NSAIDs in pancreas carcinoma BXPC3, glioblastoma A172, colon carcinoma DLD-1, oral squamous SAS, and acute myelocytic leukemia HL60 cells (Fig. 2). Our previous time-course experiments demonstrated that the sulindac-mediated ROS generation

Fig. 2. ROS generation. Carboxy-H$_2$DCFDA fluorescent signals after incubation with 0.2 mM SUL, SUD, SUF or 0.1 mM NS-398 or Piroxicam (PIRO) for 12 h in the indicated cells. Numbers indicate intracellular mean fluorescence intensities (MFIs).
was first detectable as early as 6 hours after exposure, peaked at 18 hours and declined gradually (Minami et al., 2005). Therefore, we measured ROS levels at 12 hours after exposure. ROS generation was greatest with sulindac sulfide (SUD), while sulindac (SUL) and sulindac sulfone (SUF) exhibited comparable effects on ROS generation. Importantly, SUF, which does not inhibit COX enzymatic activity, induced abundant ROS generation, indicating that NSAID-mediated ROS generation may be COX-independent. In addition, SUD, the strongest inhibitor of NF-κB (Yamamoto et al., 1999), induced ROS generation at the highest levels, which suggests that NF-κB inhibition enhances ROS generation. Indeed, there are many reports showing that NF-κB inhibits oxidative stress (Mercurio and Manning, 1999). In addition, SUD more strongly induced ROS generation in pancreas BXPC3 and colon DLD-1 cells than in other cancer cells. Consistent with this, there is a previous report that colon has lower antioxidant capacity (Blau et al., 1999). This suggests that SUD may be more potent against these adenocarcinomas than other cell types. Interestingly, the COX-2 non-selective inhibitor piroxicam and the COX-2 selective inhibitor NS-398 (Warner et al., 1999) are much weaker inducers of ROS generation than sulindac and its metabolites. Thus, among NSAIDs, sulindac and its metabolites are somehow highly potent ROS inducers. Considering that SUF has no COX-inhibitory effect, but is a strong ROS inducer, this agent or its modified derivatives may be good candidates as NSAIDs-related anticancer agents. Currently, there are several attempts to establish SUF derivatives, such as OSI-461, OSIP4866823 and OSIP487703 (Xiao et al., 2006) therapeutically. Interestingly, these drugs strongly induce cell cycle arrest and exhibit a novel action in causing microtubule depolymerization. Although their in vitro effects remain to be clarified, SUF derivatives are obviously important candidates as anticancer agents.

**ROS generation and DNA damage**

It is important to know the biological effects of increased ROS generation in NSAIDs-treated cells. Oxidative stress damages DNA (Sekiguchi and Tsuchuki, 2002), with accumulation of 8-hydroxy-2′-deoxyguanosine, 8-OHdG (Helbock et al., 1999). We previously demonstrated that sulindac weakly induces accumulation of 8-OHdG (Minami et al., 2005), and suggested a close relationship with its anticancer effect. Possibly extensive oxidative stress may produce greater DNA damage than is tolerable in each cell and thereby induces apoptosis. In contrast, aspirin has been reported to inhibit oxidative DNA strand breaks (Hue and Li 2002). As described above, sulindac increases ROS generation first, but thereafter activates scavenger enzymes in doses that are tolerated by the treated cells. From these observations, we suggest that aspirin-primed cells may have activated scavenger enzymes, which can inhibit oxidative DNA damage caused by other stimuli.

Although there is little information about lipid oxidation-mediated apoptosis after NSAIDs treatment, this may result in MPT and influx of calcium, thereby directly inducing apoptotic signals. This mechanism could also be involved in NSAIDs-mediated apoptosis.

**Combination therapy**

Human epidemiological studies, animal models, and *in vitro* experiments indicate that NSAIDs reduce the risk of colorectal cancer, and induce regression of colorectal adenomas in patients with familial adenomatous polyposis (Janne and Mayer, 2000; Bresalier, 2002). However, NSAIDs cannot be used as a single anticancer agent because of their weak activity and are likely to be more useful in combination with other agents. We previously demonstrated that combination of SUL with the proteasome inhibitor Bortezomib augments its anticancer effects in colon cancer cells (Minami et al., 2005). We conclude that the augmented effects totally depend upon oxidative stress, since the effects are barely detected when cells are pretreated with the antioxidant L-NAC. Importantly, sulindac elevates ROS generation 6 h after exposure, but the elevation declines after 18 h, and the time-course is strikingly different from that of Bortezomib-mediated ROS generation (Yu et al., 2004), which gradually increases after 24 h. Thus, their combination induces more persistent ROS generation than does individual treatment. This persistent oxidative stress may be crucial for maximal effectiveness.

As described above, sulindac and its metabolites inhibit NF-κB-mediated signals (Yamamoto et al., 1999). Based on this activity, many papers describe its therapeutic efficacy when used in combination with other agents. We and others demonstrated that sulindac augments the apoptotic potential of tumor necrosis factor-alpha (Giardina et al., 1999; Yasui et al., 2003). Similarly, it augments the anticancer activities of thalidomide or parthenolide (Verheul et al., 1999; Yip-Schneider et al., 2005). These results encourage us to further investigate their combined effects *in vivo*.

In conclusion, NSAIDs, especially sulindac and its metabolites, are strong ROS-inducing agents with anticancer activity. Although NSAIDs are long-established and commonly used medications, their use as combination partners of classical anticancer agents is very new and many things remain to be clarified. Considering that cancer cells are under increased oxidative stress (Hileman et al., 2004) and lower antioxidant activity, NSAIDs may greatly improve anticancer strategies.

Acknowledgements. We are grateful to Dr. Peter M Olley (Sapporo Medical University School of Medicine) for help with preparation of this manuscript. Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (M. A. and K. I.).
NSAID and oxidative stress

References


Accepted September 25, 2006