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Role of Thiocyanate, Bromide and Hypobromous Acid in Hydrogen Peroxide-induced Apoptosis

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We have previously reported that H_2O_2-induced apoptosis in HL-60 human leukemia cells takes place in the presence of chloride, requires myeloperoxidase (MPO), and occurs through oxidative reactions involving hypochlorous acid and chloramines. We now report that when chloride is replaced by the pseudohalide thiocyanate, there is little or no H_2O_2-induced apoptosis. Furthermore, thiocyanate inhibits H_2O_2-induced apoptosis when chloride is present at physiological concentrations, and this occurs at thiocyanate concentrations that are present in human serum and saliva. In contrast, bromide can substitute for chloride in H_2O_2-induced apoptosis, but results in a lower percent of the cells induced into apoptosis. Hypobromous acid is likely a short-lived intermediate in this H_2O_2/MPO/bromide apoptosis, and reagent hypobromous acid and bromamines induce apoptosis in HL-60 cells. We conclude that the physiologic concentrations of thiocyanate found in human plasma could modulate the cytototoxicity of H_2O_2 and its resulting highly toxic MPO-generated hypochlorous acid by competing with chloride for MPO. Furthermore, the oxidative products of the reaction of thiocyanate with MPO are relatively innocuous for human leukemic cells in culture. In contrast, bromide can support H_2O_2/MPO/halide apoptosis, but is less potent than chloride and it has no effect in the presence of physiological levels of chloride.

Keywords: Thiocyanate; Bromide; Hypobromous acid; Hydrogen peroxide; Apoptosis; Myeloperoxidase

INTRODUCTION

We have previously reported that H_2O_2-induced apoptosis in HL-60 cells requires myeloperoxidase (MPO) and Cl^-.[1] Hypochlorous acid (HOCl), a product of the oxidation of Cl^- by MPO/H_2O_2, and chloramines generated by the subsequent reaction of HOCl with amines are intermediates in the reaction.[2,3]

Thiocyanate (SCN^-) is also an important substrate of MPO and eosinophil peroxidase.[4] van Dalen *et al.*, showed that SCN^- competes well with Cl^- as a physiological substrate for MPO.[5] For example, in the presence of 100 mM Cl^-, the rate of MPO-catalyzed disappearance of H_2O_2 doubles when 100 μM thiocyanate is added. Furthermore, at physiological concentrations of both halides, approximately half of the H_2O_2 is converted into hypothiocyanite ($OSCN^-$)[5] or its conjugate acid hypothiocyanous acid (HOSCN). HOSCN, an antimicrobial agent, is probably generated in leukocytes.[6] However, HOSCN is a relatively mild oxidant for mammalian cells compared to HOCl and HOBr as measured by oxidative inactivation of ATPase.[7]

MPO can catalyze the oxidation of Br^- by H_2O_2 to hypobromous acid (HOBr).[6,8] In fact, HOBr is more potent on a molar basis than HOCl in lysing erythrocytes,[9] damaging liposome targets,[10] and in reacting with unsaturated fatty acids.[11] Much of our knowledge of HOBr is derived from studies of eosinophils. HOBr is generated by human eosinophils from their characteristic eosinophil peroxidase.[12] In contrast to MPO, which prefers to utilize Cl^-

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at plasma concentrations, eosinophil peroxidase preferentially oxidizes Br^-.[12,13] HOBr likely has importance in human disease. For example, HOBr generated by eosinophils may play a role in asthma.[14]

Since MPO plays a fundamental role in oxidant production by some human leukemia cells, and since H_2O_2 may be an important mediator of the action of chemotherapeutic agents[15,16] and ionizing radiation,[17] we studied whether the halides SCN^- and Br^- could substitute for or modulate Cl^- in apoptosis induced by the addition of H_2O_2 to the MPO-containing HL-60 human leukemia cell line. We found that Br^- supports H_2O_2-induced apoptosis in this cell line suggesting that HOBr is a likely intermediate oxidative mediator. In contrast, SCN^- was not able to support H_2O_2-induced apoptosis. Most importantly, SCN^- but not Br^- inhibited H_2O_2-induced apoptosis in the presence of physiological concentrations of Cl^-. Furthermore, we found a continuum of apoptotic vs. necrotic H_2O_2 cytotoxic activity supported by the halides that may relate in part to the oxidation potential of their metabolic products.

MATERIALS AND METHODS

Cells and General Procedures

HL-60 human leukemia cells (obtained from American Type Tissue Culture, Rockville, MD) were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and supplemented with 1.5 mM L-glutamine, 85 units ml^{-1} penicillin and 85 $\mu g\,ml^{-1}$ streptomycin (Gibco BRL, Life Technologies, Grand Island, NY). Log phase cells were washed and placed in medium and the cell density was adjusted to $0.5 \times 10^6\,ml^{-1}$ for apoptosis experiments. Cell counts were determined with a Coulter Model Z_f cell counter (Coulter, Inc., Hialeah, FL) and viable counts using trypan blue dye exclusion. HL-60 cells were exposed to H_2O_2 or HOBr in RPMI 1640 with 10% FBS (referred to hereafter as "full medium") at various concentrations and times. The cells were then incubated for 24 h before extraction of DNA. For the halide experiments, HL-60 cells were exposed to the oxidant in the halide-adjusted medium described below, then catalase (500 or 1000 units ml^{-1}) and full medium containing methionine (50 mM) were added to stop further oxidation. The cells were then pelleted, washed and resuspended in full medium for a 24 h incubation prior to DNA extraction. Apoptosis positive controls with ultraviolet light were carried out at 302 nm for 5 min.

Preparation of Halide-free and Halide-rich Medium

Halide-free medium, at pH 7.4, contained 140 mM sodium gluconate, 5 mM potassium gluconate, 1 mM $MgSO_4$, 1.8 mM calcium acetate, 10 mM glucose, and 10 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid). The effect of halides was studied in the same medium modified by replacing sodium gluconate with NaSCN, NaBr or NaCl at the same concentration level of 140 mM (or in the concentration studies, as shown) and 0.1% (w/v) bovine serum albumin.[18] The medium formulations were not completely Cl^--free due to its presence as a trace contaminant in the reagents. For example, medium containing 140 mM NaBr (Fisher Scientific Co., Fair Lawn, NJ) also contained 10.8 μM Cl^-. Experiments that did not involve variation of the halide concentration were performed in full medium, which contains 108 mM Cl^-.

Preparation of HOBr Reagent and Bromamines

To produce authentic HOBr, NaBr (2.27 g) was added to 10 ml of 740 mM NaOCl while stirring. The concentration of the resultant HOBr was confirmed spectrophotometrically $\varepsilon_{329} = 326\,M^{-1}\,cm^{-1}$.[19,20] In this paper HOBr is used to include the equilibrium mixture of the acid form with its conjugate base $HOBr/OBr^-$. That the conversion of HOCl into HOBr was complete was verified spectrophotometrically by measuring absorbances at 329 nm (generation of HOBr) and 292 nm ($\varepsilon_{292} = 350\,M^{-1}\,cm^{-1}$) (loss of HOCl).

Determination of Apoptosis

Apoptosis was determined by DNA fragmentation and agarose gel electrophoresis using standard techniques as previously described.[1] Shown are representative gels from 2–5 independent experiments. To confirm and quantify the apoptosis, we used a complementary method, morphology of cytospin preparations. Following experimental incubations, cytospin preparations were made as previously described.[1] Dried slide preparations were then stained with Wright's stain and morphological evaluations made by light microscopy. Triplicate 100-cell counts on different samples were done. The specific morphologic criteria of apoptosis are described in an earlier publication.[1] Briefly, cells were considered apoptotic if there was condensed nuclear chromatin (including chromatin crescents along the nuclear envelope and apoptotic bodies), or in the absence of those features, had both cell shrinkage and definite membrane blebbing.

Preparation of Authentic Bromamines and 2-nitro-5-thiobenzoate (TNB) Reactivity

Bromamines of taurine, glutamine, and ammonium monobromamine were prepared by making a 200 mM solution of the amine in H_2O. Then 20 ml of this amine solution was cooled to 5°C, and 2.7 ml

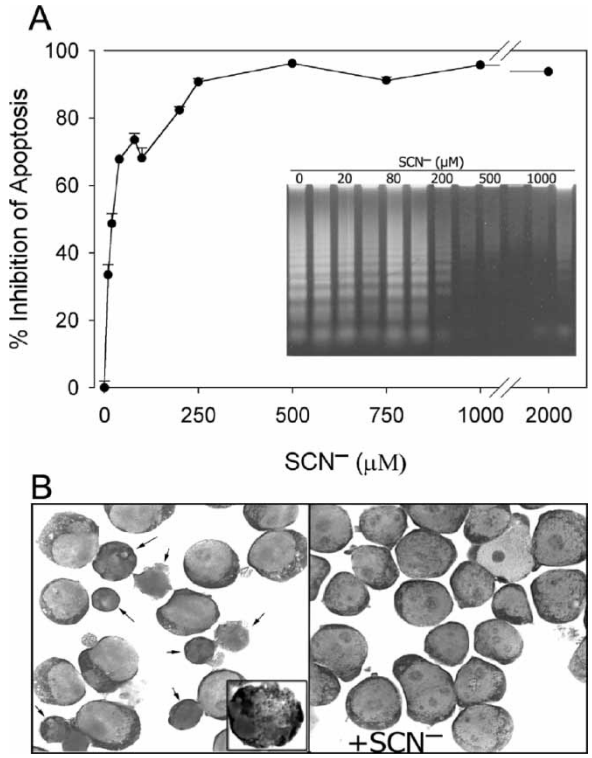

FIGURE 1 Thiocyanate inhibits H_2O_2-apoptosis. HL-60 cells were incubated with 20 μM H_2O_2 in a medium containing physiological concentrations of Cl^- (100 mM), Br^- (50 μM), I^- (0.1 μM) and gluconate (40 mM). Then increasing concentrations of SCN^- were added. After 15 min at 37°, catalase (500 units ml^{-1}) and methionine (50 mM) were added, cells were harvested, resuspended in fresh medium then incubated for 24 h before apoptosis was determined by morphology of Wright's stained cytospin preparations (A) apoptosis in the absence of SCN^- was 23 ± 2%. Inset shows apoptosis as measured by a complementary method, DNA fragmentation. For these studies, DNA was isolated, separated on a 1% agarose gel for 2.5 h at 50 V (2 μg per lane) and stained with ethidium bromide. The lanes from left to right are from samples with 0, 10, 20, 40, 80, 100, 200, 250, 500, 750, 1000, 2000 μM SCN^-. (B) left panel shows a typical bright field microscope view of H_2O_2-induced apoptosis of the HL-60 cells after Wright's staining in the absence of added SCN^- with one apoptotic cell enlarged to see nuclear apoptotic bodies. Right panel shows a typical field of cells exposed to H_2O_2 under identical conditions in the presence of 1000 μM SCN^-.

of NaOBr/OBr^- stock (90 mM) was added drop wise over 1 min while stirring with a magnetic stir bar (approximate mole ratio of amino acid–HOBr 2.2:1). Bromamine solution (125 μl) was placed into 5 ml of water, mixed and then scanned on a Hewlett Packard diode array spectrophotometer Model 8453 to monitor bromamines and HOBr/OBr^-. Spectra were analyzed using UV–Visible ChemStation software.

TNB reactivity was determined using the method of Kettle and Winterbourn measuring absorbance at 412 nm[21] and absoption coefficient of Eyer *et al.*[22]

Statistical Analysis

The results are expressed as mean ± SE. Significant differences were evaluated with one-way analysis of variance, Dunnett's multiple comparison procedure or the unpaired Student's *t*-test. All statistical tests were carried out at the 5% level of significance.

RESULTS

Thiocyanate Inhibits H_2O_2-induced Apoptosis in the Presence of Chloride

Since SCN^- and Cl^- are competing substrates for MPO,[5] we studied the effect of SCN^- on H_2O_2-induced apoptosis in the presence of physiological levels of Cl^-, and other halides. Figure 1A shows that SCN^- inhibits H_2O_2-induced apoptosis in a concentration dependent manner with the effect reaching a plateau at 200–250 μM. These results by morphology were confirmed biochemically using internucleosomal DNA banding (Fig. 1A inset). Figure 1B (left panel) shows the small apoptotic cells induced when no SCN^- was added. In contrast, these apoptotic cells are not present in the preparations containing 1 mM SCN^- (Fig. 1B, right panel).

Thiocyanate Increases 2-nitro-5-thiobenzoate (TNB) Oxidation

Since SCN^- inhibits apoptosis, we tested whether SCN^- alters the oxidation of TNB by cells. The hypohalous acid products of halide oxidation and their chloramine derivatives react with TNB. The results are shown in Fig. 2. There was an SCN^- concentration-dependent increase in TNB oxidation to 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) that was dependent upon the presence of the MPO-rich HL-60 cells and occurred in the presence

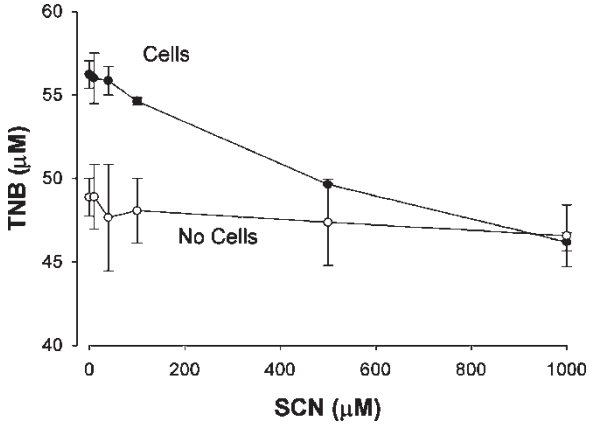

FIGURE 2 Thiocyanate increases oxidation of TNB. HL-60 cells (5×10^6 ml^{-1}) were incubated in gluconate buffer (containing 100 mM Cl^- and 50 μM Br^-), pH 7.4 with 20 μM H_2O_2. No BSA was added. After 30 min at room temperature, TNB was determined as a change in absorbance at 412 nm. The initial concentration of TNB was 80 μM. Shown is the decrease in TNB as it is oxidized to DTNB. Values are the mean and SEM of 9–10 replicates in two separate experiments.

of physiological concentrations of Cl^- and Br^-. This indicates that although increasing concentrations of SCN^- inhibit apoptosis, there is no similar inhibition of TNB oxidation to explain it.

Thiocyanate does not Support H_2O_2-induced Apoptosis or Necrosis in the Absence of Chloride

Since the pseudohalide SCN^- is a major substrate for MPO,[5] we incubated HL-60 cells for varying times in a Cl^- free medium containing NaSCN (140 mM). There was little apoptosis at 0, 5, 10 and 15 min of exposure to 20 μM H_2O_2 by morphology (2% ± 2, 3% ± 1, 3% ± 1, 3% ± 2, respectively). Likewise, there was no banding on DNA fragmentation analysis (not shown). The majority of the cells were intact and viable with no evidence of either necrosis or apoptosis under these conditions of time and concentration.

Bromide Supports H_2O_2-induced Apoptosis

In order to determine if Br^- can substitute for Cl^- in supporting H_2O_2-induced apoptosis by the action of H_2O_2/MPO/ halide, we incubated HL-60 cells with H_2O_2 for 15 min in medium with Br^- substituted for Cl^-. As can be seen in Fig. 3, Br^- can substitute for Cl^- and support apoptosis. By morphology an increase in apoptotic cells could be detected after being exposed to 15–20 μM H_2O_2. Apoptosis was evident by DNA fragmentation at 5, 10 and 20 μM H_2O_2 (Fig. 3 inset). However, above 20 μM, apoptosis declined, and necrosis appeared to be the predominant mode of cell death.

H_2O_2-induced Apoptosis is a Function of Br^- Concentration and Time

In order to further confirm the effect of Br^-, and to determine if apoptosis induced by H_2O_2 is dependent upon Br^- concentration, we incubated HL-60 cells in Cl^--free medium containing increasing concentrations of Br^- and determined the extent of apoptosis. As can be seen in Fig. 4, the percentage of apoptosis (by morphology) induced by H_2O_2 increased in a Br^- concentration-dependent manner up to 0.5 mM of Br^- then reached a plateau. Figure 4 inset shows DNA fragmentation at the same Br^- concentrations and confirms the morphology results. Concentrations of Br^- in human whole blood are 16–101 μM,[23] therefore, the concentrations of Br^- used here are in the physiological range.

We also carried out a time course of H_2O_2-induced apoptosis in Br^--containing medium. Cells were exposed to H_2O_2, then washed and incubated in full medium for 24 h before DNA was extracted. Incubation with H_2O_2 for about 10 min was required

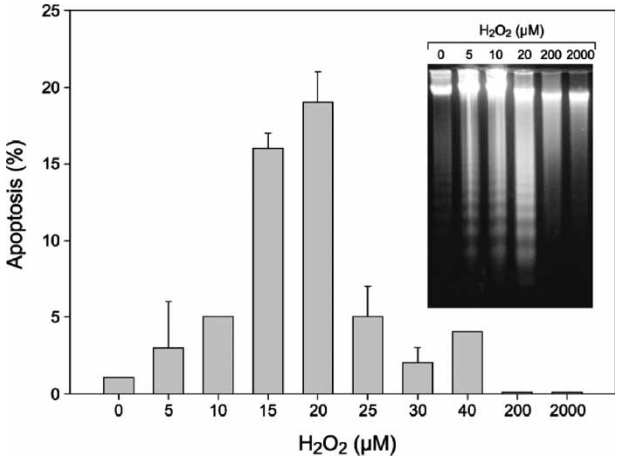

FIGURE 3 Bromide-rich medium supports H_2O_2-induced apoptosis. HL-60 cells were treated with H_2O_2 for 15 min in Cl^--free medium containing 140 mM Br^-. Catalase (1000 units ml^{-1}) was added to remove residual H_2O_2 as well as methionine (50 mM), the cells were then centrifuged and resuspended in fresh medium. The cells were incubated for 24 h then apoptosis was determined by morphology of Wright's stained cytospin preparations. The overall test of differences among the concentrations was statistically significant ($p < 0.0001$ by one-way analysis of variance); concentrations 15 and 20 μM were significantly different from the control (concentration = 0) by Dunnett's multiple comparison procedure. Inset shows apoptosis as measured by a complementary method, DNA fragmentation. For these studies, DNA was isolated, separated on a 1% agarose gel for 2.5 h at 50 V (2 μg per lane) and stained with ethidium bromide.

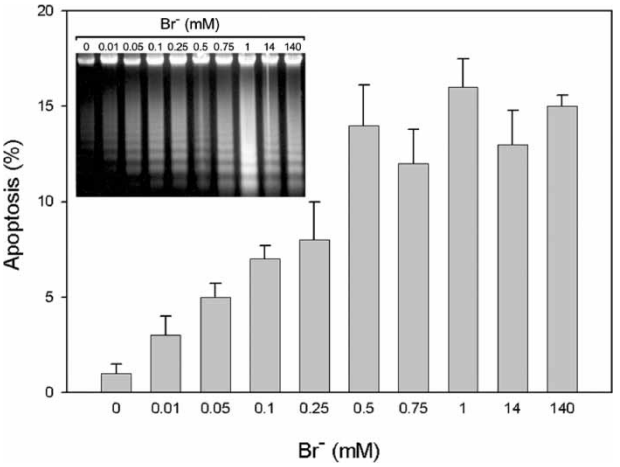

FIGURE 4 Bromide concentration-dependence of H_2O_2-induced apoptosis in Cl^--free medium. HL-60 cells were treated with 20 μM H_2O_2 for 15 min in Cl^--free medium containing increasing concentrations of Br^-. Catalase (1000 units ml^{-1}) to remove residual H_2O_2 and methionine (50 mM) were added, the cells were centrifuged and resuspended in fresh medium. The cells were incubated for 24 h then apoptosis was determined by morphology of Wright's stained cytospin preparations and by DNA fragmentation (Inset). For the latter, DNA was isolated, separated on a 1% agarose gel for 2.5 h at 50 V (2 μg per lane) and stained with ethidium bromide. The overall test of differences among the concentrations was statistically significant ($p < 0.0001$ by one-way analysis of variance); concentrations 0.1, 0.25, 0.5, 0.75, 1, 14 and 140 mM were significantly different from the control (concentration = 0) by Dunnett's multiple comparison procedure.

to reach peak levels of apoptosis at 20 μM H_2O_2 (data not shown).

Incubation in Halide-free Medium does not Stimulate or Inhibit Apoptosis

To demonstrate that incubation in halide-free medium does not stimulate apoptosis independent of oxidant exposure, or conversely, preclude subsequent H_2O_2-induced apoptosis, HL-60 cells were preincubated in halide-free medium or in medium containing Cl^-, Br^- or SCN^- (data not shown). Exposure to these media neither stimulated nor inhibited apoptosis when the cells were subsequently placed into full medium to which H_2O_2 was added. In addition, gluconate does not induce or inhibit H_2O_2-induced apoptosis.

Bromide has no Effect on Apoptosis in the Presence of Chloride

To examine the effect of Br^- on H_2O_2-induced apoptosis in the presence of Cl^-, we treated HL-60 cells (0.5×10^6 ml^{-1}) with 20 μM H_2O_2 for 15 min in medium containing increasing concentrations of Br^- in the presence of 140 mM Cl^- (data not shown). The Br^- concentrations were chosen to encompass the physiologic range for human blood. There was no effect of [Br^-] in the range of 0–100 μM. To further explore the effect of added Br^-, we did multiple replicates ($n = 7$ experiments) with and without Br^- in the presence of 140 mM Cl^-. This confirmed that there is no effect of 10 μM Br^- (38 ± 2%) vs. 0 μM Br^- (37 ± 2%) on H_2O_2-induced apoptosis performed in the presence of Cl^-. Taken together these two studies indicate that there is no detectable inhibitory or additive effect of Br^- at any concentration when Cl^- is at 140 mM concentration. This observation stands in contrast to the studies with SCN^- which showed inhibition of apoptosis under these conditions.

Authentic HOBr Induces Apoptosis

Since HOCl is an intermediate mediator of H_2O_2-induced apoptosis in HL-60 cells when Cl^- is the predominant halide present in the medium,[2] and because HOBr is likely formed when Br^- is present, we assessed whether HOBr causes apoptosis. We incubated HL-60 cells with authentic HOBr for 15 min in full medium in the absence of H_2O_2, and we found apoptosis beginning at about 100 μM (Fig. 5). Therefore, authentic HOBr is capable of stimulating apoptosis in this human leukemia cell in full medium. HOBr also induced necrosis. There was trace necrosis beginning at about 100 μM, and at higher concentrations it increasingly became the predominant form of cell death.

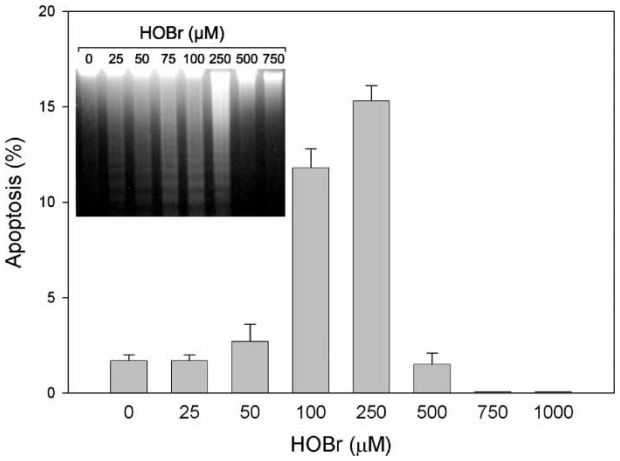

FIGURE 5 HOBr induces apoptosis in HL-60 cells. HL-60 cells were treated with various concentrations of authentic HOBr for 15 min in full medium, then fresh medium containing 50 mM methionine was added, cells centrifuged and resuspended in fresh full medium for an additional 24 h before determination of apoptosis as measured by morphology of Wright's-stained cytospin preparations. The overall test of differences among the HOBr concentrations was statistically significant ($p = 0.0001$ by one-way analysis of variance); concentrations of 100 and 250 μM were significantly different from the control by Dunnett's multiple comparison procedure. Inset shows apoptosis as measured by a complementary method, DNA fragmentation. For these studies, cells were treated with increasing concentrations of HOBr for 15 min, and genomic DNA isolated, separated and stained with ethidium bromide.

The dose-effect curves for the induction of apoptosis are different for HOCl and HOBr. We have previously reported that HL-60 cells exposed to HOCl for 24 h at 250, 500, 1000 and 2000 μM have apoptosis of 4, 11, 15 and 50%, respectively.[2] For comparison, when HL-60 cells were exposed to HOBr under the same conditions, the percentages of apoptosis were only 1, 3, 7 and 11%.

HOBr Fails to Induce Apoptosis in Amine-free Medium

To further explore the possibility that bromamines formed from the reaction of HOBr and amines in the full medium may induce apoptosis in the HL-60 cells, we studied apoptosis in Hanks' balanced salt solution (HBSS), which does not contain amino acids or amines with which HOBr could react to form bromamines or other compounds. HL-60 cells (0.5×10^6 cells ml^{-1}) in HBSS were exposed to HOBr for 5–10 min, then the cells removed by centrifugation (250 *g*, 5 min), and resuspended in full medium. They were then incubated another 24 h before determination of apoptosis. There was no concentration that produced appreciable apoptosis even at short times of exposure. There was a low level of apoptosis of about 5% at 5 μM HOBr (Fig. 6); at all other concentrations, there was little or none. Beginning at 5 μM HOBr there was about 20%

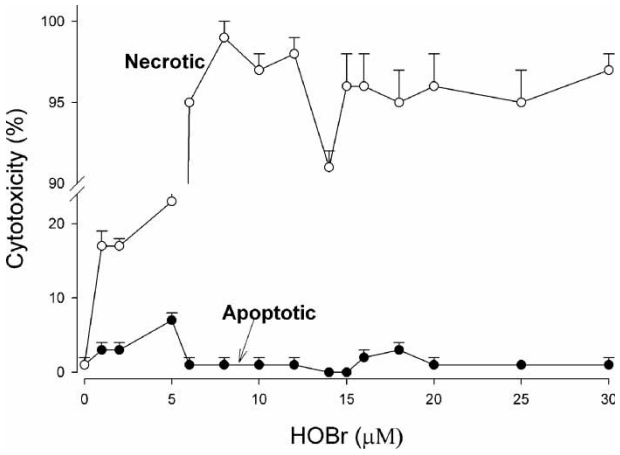

FIGURE 6 HOBr fails to induce appreciable apoptosis in amine-free HBSS. HL-60 cells were exposed to HOBr at various concentrations for 5–10 min in HBSS, and then full medium containing 50 mM methionine was added to remove the HOBr. The cells were then centrifuged 250*g* for 5 min, resuspended in full medium and incubated for 24 h and apoptosis determined.

necrosis that reached 100% at higher concentrations (not shown).

Concentration-dependence of Apoptosis Induced by Taurine Bromamine

We next examined whether bromamines can induce apoptosis. HL-60 cells were treated with increasing concentrations of taurine bromamine in full medium. Apoptosis was detected at 250–750 μM and peaked at 500 μM (41 ± 3%). DNA fragmentation confirmed the result. We found that glutamine bromamine and ammonium bromamine also induce apoptosis at similar concentrations (data not shown). This indicates that bromamines, if formed, could play a role in H_2O_2-induced apoptosis.

DISCUSSION

We have shown an apoptotic-protective effect of adding SCN^- to a medium containing H_2O_2 and physiological concentrations of halides, including Cl^-. It is possible to explain this protection based on reaction rates of halides with MPO/H_2O_2 and the differing toxicity of resultant hypohalous acids. Even though the normal plasma concentration of SCN^- (34 μM in nonsmokers, 122 μM in smokers[24]) is ~1000-times less than that of Cl^- (100 mM), SCN^- has a specificity constant for MPO that is 730-times higher.[5] It can be calculated that at relevant concentration of reactants, the amount of the respective oxidation products HOCl and HOSCN will be similar.[4,25] So in effect SCN^- competes for and may divert the MPO/H_2O_2 oxidation away from Cl^- and decreases the production of the more toxic HOCl. The HOSCN that is produced is a weaker mainly thio-reactive molecule and is less damaging to nucleated[4] and non-nucleated mammalian cells.[26] McCormick *et al.*, showed that SCN^- blocks the formation of hydroxyl radical from H_2O_2, eosinophil peroxidase and Br^-.[27] Slungaard *et al.*, found that SCN^- at 3.3–10 μM eliminated the Br^--dependent toxicity of eosinophil peroxidase for aortic endothelial cells and rat hearts.[4] Similarly, HOSCN/$OSCN^-$ slows the growth of but does not kill bacteria.[28] This effect of SCN^- on halide oxidation by MPO/H_2O_2 is even greater in human saliva where the concentration of SCN^- is 0.83 mM.[29] It should be possible to exploit the unique properties of NaSCN (low cytotoxicity, preferential reaction with MPO) to suppress production of more cytotoxic metabolites of MPO derived from Cl^- even using physiological concentrations of the compound.

In our experiments with MPO-rich cells, H_2O_2 and physiological concentrations of Cl^-, we found that as the concentration of SCN^- increased, the oxidation of TNB to the colorless DTNB increased (Fig. 2). This is likely due to the fact that the major product of MPO-catalyzed oxidation of SCN^- is HOSCN/$OSCN^-$, which like HOCl oxidizes TNB.[28] Since SCN^- reacts with MPO Compound I at a rate 2–3 orders of magnitude faster than Cl^-,[5,6] there is an increasing amount of $OSCN^-$ formed in the experimental system as the SCN^- concentration is raised. For example, at 1000 μM SCN^- the concentration of Cl^- is only 100-fold greater than the concentration of SCN^- so given the relative reaction rates, $OSCN^-$ would become a predominant product, and the rate of oxidation of TNB would increase above that resulting from the Cl^- alone. In that regard, van Dalen *et al.*, have shown in a cell-free system with MPO that 25 μM H_2O_2 generates 2-fold more oxidant in the presence of 100 μM each of Cl^- plus SCN^- as compared to 100 μM Cl^-.[5]

We found that when Cl^- was replaced with SCN^-, there was no appreciable apoptosis in the HL-60 cell line and little toxicity of any kind. This pseudohalide is a physiologically relevant MPO substrate and a preferred electron donor for MPO Compound I.[5] The product of its oxidation is thought to be HOSCN.[30] Our observations suggest that HOSCN is a weak stimulus of apoptosis in the HL-60 line. In this regard it is known that HOSCN is less reactive than HOCl,[4,6,27] and this supports the observation of our study that it is not an apoptosis inducer. This may be because the products of the reaction in the full medium of this primarily sulfhydryl-reactive oxidant are less reactive and toxic to mammalian cells.

In the presence of physiologic concentrations of Cl^-, Br^- had no additional effect on H_2O_2-induced apoptosis. This suggests that in the blood, for example, Br^- may not play a role in oxidant-induced

apoptosis. However, it is known that myeloperoxidase can generate reactive brominating species in reactions involving HOCl, Br^- and the interhalogen gas bromine chloride.[31] Therefore, in the presence of both Cl^- and Br^-, BrCl may allow the interchange between HOCl and HOBr and provide a rationale for a role of HOBr. Furthermore, the relative concentrations of the halides vary in the body, and at selected sites Br^- may contribute to programmed cell death. For example, it is known that there is an active secretion of Br^- into the lumen of excised lungs.[32] Furthermore, in aqueous zwitterionic micelles the surface concentration of Br^- becomes higher than that of Cl^-, showing that binding is anion selective under certain conditions[33] that might exist in selected tissues of the intact organism.

HOBr is capable of inducing apoptosis as demonstrated by adding reagent HOBr to full medium, and it is likely to be the mediator of apoptotic cell death when HL-60 cells are placed in a Br^--enriched Cl^--free medium in the presence of H_2O_2. However, authentic HOBr induces apoptosis less efficiently than we found previously for HOCl.[2,34] Also HOBr causes cellular necrosis at lower concentrations. Differences in chemical reactivity between HOCl and HOBr, and between their reaction products may explain the disparity in percent of apoptosis induced by these reagents, including the limited apoptosis by authentic HOBr. The HOBr may react with other compounds in the medium leaving less oxidant for the production of the mediators that induce apoptosis.

We have previously identified HOCl and a species with similarities to chloramines as intermediates in the apoptosis induced by H_2O_2 and Cl^-.[2] In our current experiments, the lack of apoptosis and persistence of unreacted HOBr when HOBr is added to amine-free medium suggests that bromamines may mediate H_2O_2-induced apoptosis. The concentrations of bromamine (see "Results" Section) and chloramine[2] needed to induce minimal apoptosis are similar. However, we have no evidence of bromamine formation. This differs from the previously reported experiments done in Cl^--containing medium in which a species with the absorbance of chloramines was identified.[2] It is possible that bromamines are formed and rapidly react to produce some metabolic product such as aldehydes.[35]

HEPES buffer was contained in the incubation medium of all of our experiments in which the halide concentration was adjusted. This piperazine-ring containing Good's buffer can be oxidized to a radical, and under some conditions is a hydroxyl radical scavenging agent.[36] Because of this, we cannot rule out the possibility that some results in those experiments carried out in medium containing HEPES (Figs. 1–4) could have been affected by its presence. However, we doubt that the buffer had any effect that would modify our conclusions. There is no evidence that the HEPES itself stimulated apoptosis since there was only the expected level of baseline apoptosis when the buffer was present in the absence of H_2O_2 (Fig. 3) or in the presence of H_2O_2 and absence of Br^- (Fig. 4). Likewise, there was no inhibition of apoptosis at zero concentrations of SCN^- in the presence of HEPES (Fig. 1). It is reassuring that the current observations were quantitatively and qualitatively consistent with our previous work on oxidant-induced apoptosis, all done in the absence of HEPES.[2,34] Furthermore, in all the experiments containing the buffer, we saw a concentration-dependent effect of the oxidant (Fig. 3) or halide (Figs. 1, 2 and 4) on apoptosis even though the concentration of HEPES remained the same. It is that change upon which our conclusions are based. A similar argument pertains to the presence of thiols in albumin that are known to be a target for oxidation by HOCl and chloramines.[37]

Our data suggest that there is a halide oxidation hierarchy for the induction of apoptosis that applies to the MPO-containing HL-60 cells exposed to H_2O_2. Br^- is capable of producing apoptosis, but it is less than we have previously reported for Cl^-, and at most concentrations, there is more necrosis than apoptosis. In the presence of SCN^-, H_2O_2 has little toxicity of any type in Cl^--free medium. Cl^- is the preferred substrate for inducing apoptosis.[2] Therefore, the order of capacity for the induction of apoptosis is $Cl^- > Br^- \gg SCN^-$. Each anion, except perhaps SCN^-, can support H_2O_2-induced necrotic cell death if the concentration is high.

Figure 7 is a diagram summarizing our current picture on mediators, showing the relationship of specific halides, metabolic pathways and cytotoxicity. The left side of the diagram summarizes our suggestion on the mediators of H_2O_2-induced cell death as a function of the halide present. It is known that when Cl^- is oxidized to HOCl by H_2O_2/MPO, it can subsequently react with amines to form chloramines.[38,39] Chloramines appear to be an important mediator in the H_2O_2-induced apoptosis pathway in HL-60 cells.[2] Br^- also supports H_2O_2 cytotoxicity, and there are differences in the process compared to Cl^-. Our present data demonstrate that when Br^- is substituted for Cl^-, considerable cytotoxicity still occurs, but there is less apoptosis. We postulate that Br^- is oxidized to HOBr, which is a short-lived chemically active intermediate that induces some apoptosis, but primarily necrosis. The right side of Fig. 7 shows the type of cell death related to each specific halide as determined in the present study and in previous ones.[1,2] There is no apoptosis or necrosis in the absence of halide or when SCN^- replaces Cl^-. This diagram provides an overview of our observations. It does not include

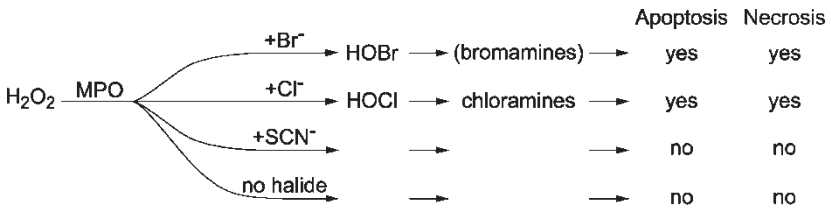

FIGURE 7 Summary diagram of metabolic pathway and cellular toxicity of specific halides with H_2O_2/MPO in the HL-60 human leukemia. The oxidative intermediates in the HL60 H_2O_2/MPO/halide system identified in this study and in our previous work[1,2] are shown. Unidentified steps and mediators are shown by arrows and blank spaces. Parentheses around bromamines indicate that the evidence for its role is indirect. Apoptosis and necrosis were estimated from experiments of this paper (Br^-, SCN^-) or our previous work for Cl^-[1,2] and are designated qualitatively as "yes," or "no." When cells were exposed to 20 μM H_2O_2 in the presence of 140 mM Cl^- there was 3, 3, 7% necrosis at 5, 10 and 15 min exposures. For comparison, studies of necrosis in the presence of 140 mM Br^- using the same time and conditions were 13, 19 and 42% necrosis, respectively. Studies with 140 mM SCN^- showed neither apoptosis nor necrosis.

variations such as observed by Vissers *et al.*[40] They have reported that chloramines block apoptosis initiated by serum deprivation and that a reducing agent such as ascorbate can abrogate that effect.

In summary, the major findings of this study are these: (1) In the presence of physiologic concentrations of Cl^-, SCN^- but not Br^- has a protective effect against H_2O_2-induced apoptosis; (2) Br^- can substitute for Cl^- in inducing apoptosis in HL-60 human leukemia cells exposed to H_2O_2. However, at similar concentrations, Br^- results in less apoptosis and more necrosis than Cl^-; (3) As previously demonstrated for HOCl, HOBr also induces apoptosis; (4) Reagent bromamines can induce apoptosis, however, we found only indirect evidence that bromamines generated from H_2O_2/MPO/Br^- are involved when Br^- is the predominant halide present; (5) SCN^- was not capable of inducing apoptosis in these cells in Cl^--free medium in the presence of H_2O_2. Overall, this report and earlier studies demonstrate that specific halides can modulate cell death events induced by relatively low levels of the oxidant H_2O_2, independent of changes in other enzymatic and antioxidant factors. These observations are important because halides are essential components in the sequence of oxidative events mediated by MPO.

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