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LUTEOLIN AND THIOSALICYLATE INHIBIT $HgCl_2$ AND THIMEROSAL-INDUCED VEGF RELEASE FROM HUMAN MAST CELLS

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$HgCl_2$ is a known environmental neurotoxin, but is also used as preservative in vaccines as thimerosal containing ethyl mercury covalently linked to thiosalicylate. We recently reported that mercury chloride ($HgCl_2$) can stimulate human mast cells to release vascular endothelial growth factor (VEGF), which is also vasoactive and pro-inflammatory. Here we show that thimerosal induces significant VEGF release from human leukemic cultured LAD2 mast cells (at 1 μM 326±12 pg/10^6 cells and 335.5±12 pg/10^6 cells at 10 μM) compared to control cells (242±21 pg/10^6 cells, n=5, $p<0.05$); this effect is weaker than that induced by $HgCl_2$ at 10 μM (448±14 pg/10^6 cells) (n=3, $p<0.05$). In view of this finding, we hypothesize that the thiosalicylate component of thimerosal may have an inhibitory effect on VEGF release. Thimerosal (10 μM) added together with the peptide Substance P (SP) at 2 μM, used as a positive control, reduced VEGF release by 90%. Methyl thiosalicylate (1 or 10 μM) added with either SP or $HgCl_2$ (10 μM) inhibited VEGF release by 100%, while sodium salicylate or ibuprofen had no effect. Pretreatment for 10 min with the flavonoid luteolin (0.1 mM) before $HgCl_2$ or thimerosal completely blocked their effect. Luteolin and methyl thiosalicylate may be useful in preventing mercury-induced toxicity.

Mercury compounds are found in fish, such as tuna, due to water pollution as well as in antiseptics, disinfectants, toothpastes, lens solutions, and various drugs such as contraceptives, fungicides, and herbicides. Ethyl mercury covalently linked to thiosalicylate, known as thimerosal, has been extensively used as a preservative in vaccines (1). Mercury is a known neurotoxin associated with neurodevelopmental defects and peripheral neuropathies. Mercury exposure causes immune, sensory, neurological, motor, and behavioral dysfunction similar to symptoms associated with autism (1).

We recently reported that $HgCl_2$ can induce release of VEGF from human mast cells. $HgCl_2$ also induces the release of histamine and cytokines, such as IL-4 and tumor necrosis factor-alpha (TNF-α) from a murine mast cell line and mouse bone marrow-derived cultured mast cells (2).

In this study, we investigated whether thimerosal could stimulate VEGF release from human mast cells, and whether its thiosalicylate component and

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the flavonoid luteolin could block this effect.

MATERIALS AND METHODS

Materials

$HgCl_2$ was obtained from Fluka Chemical Corp. (Milwaukee, WI). Thimerosal, sodium salicylate, methyl thiosalicylate, SP, ibuprofen and luteolin were obtained from Sigma-Aldrich (St. Louis, MO, USA). $HgCl_2$ was dissolved in Dulbecco's phosphate buffered saline (DPBS, GIBCO, Grand Island, NY, USA). Luteolin was dissolved in DMSO, but the final concentration was less than 0.1%. The other drugs were dissolved in distilled water on the day of the experiments.

Human mast cell culture

LAD2 human mast cells were cultured in serum-free media (StemPro-34; GIBCO, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml recombinant human Stem Cell Factor (rhSCF) (3). For optimal cell growth, LAD2 cell density was maintained between 0.5×10^6 and 1×10^6 cells/ml. Cell viability was assessed at 1 h and 24 h using trypan blue (0.3%) exclusion.

VEGF assay

LAD2 cells were washed with DPBS and suspended in complete culture medium. LAD2 cells (2×10^5 cells/well/200µl) were plated in 96-well flat bottom Falcon cell culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) and were pre-incubated for 15 min at 37°C in 5% CO_2 atmosphere. The cells were then incubated with either SP (2 µM), $HgCl_2$ (10 µM) or thimerosal (1, 10 µM) for 24 h at 37°C. Control cells were treated with equal volume of only culture medium. For the inhibition experiments, thimerosal or methyl thiosalicylate were added together with the triggers, while luteolin was added 10 min before the triggers. After the reaction time, plates were centrifuged and the supernatant was gently collected from the wells and stored at -20°C until VEGF was measured by Enzyme-Linked Immunosorbent Assay (ELISA) using a commercial kit (R&D Systems, Minneapolis, MN, USA). The minimum detectable level of VEGF was 5 pg/ml.

Statistical analysis

All conditions were performed in triplicate, and all experiments were repeated up to five times (n=3-5). Results are presented as mean±SD. Data from two conditions, such as between stimulated and control samples, were compared using the unpaired 2-tailed, Student's t-test. Significance of comparisons is denoted by $p<0.05$.

RESULTS

Effect of thimerosal on mast cell viability

LAD2 mast cells were incubated with thimerosal for 24 h in the culture medium and their viability was assessed by trypan blue exclusion. Viability was decreased by less than 10% only at thimerosal concentrations of 10 µM (n=3, Fig. 1A).

Effect of thimerosal on LAD2 mast cell VEGF release

LAD2 cells stimulated with thimerosal for 24 h released significantly more VEGF at 1 and 10

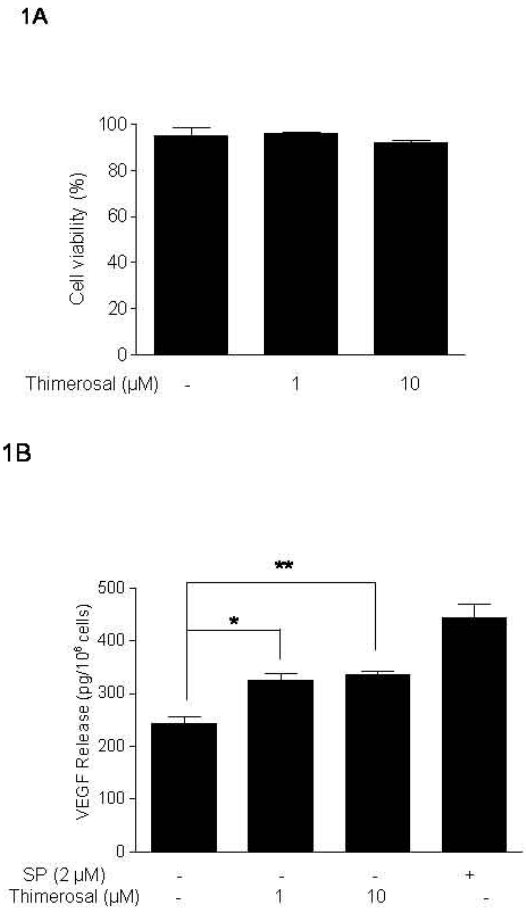

Fig 1. ***A)*** *The effect of thimerosal on mast cell viability after 24 h.* ***B)*** *Effect of thimerosal on VEGF release from LAD2 mast cells. Mast cells were incubated with thimerosal and/or SP as indicated for 24 h for VEGF release at 37°C. The cells were then centrifuged and the supernatant fluid was collected. VEGF release was assayed by ELISA (n=5, $p<0.05$).*

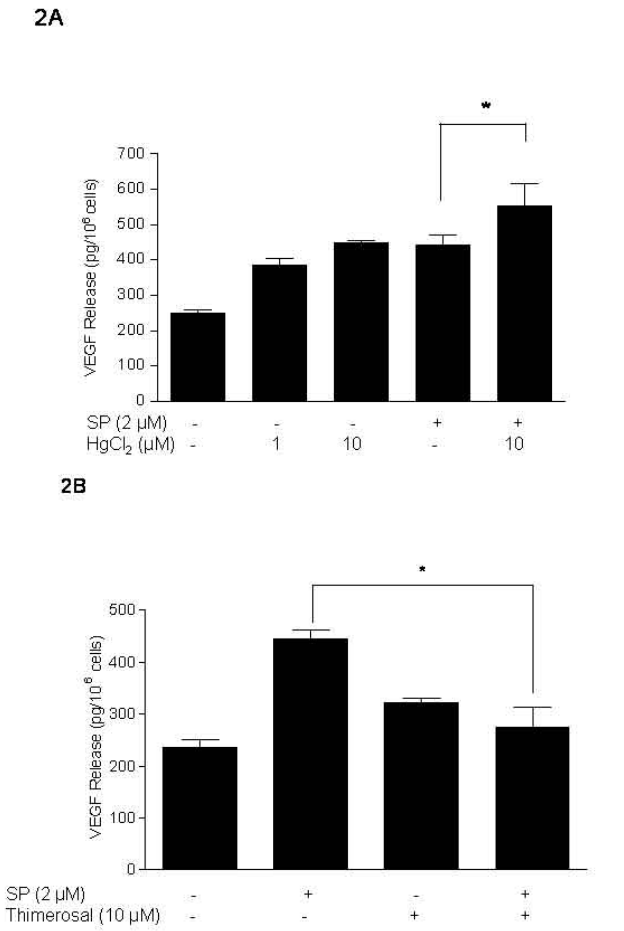

Fig 2. *Effect of (**A**) $HgCl_2$ and (**B**) thimerosal on SP-induced VEGF release from LAD2 cells. Mast cells were incubated as indicated for 24 h at 37°C. The cells were then centrifuged and the supernatant fluid was collected. VEGF release was assayed by ELISA (n=3, p<0.05).*

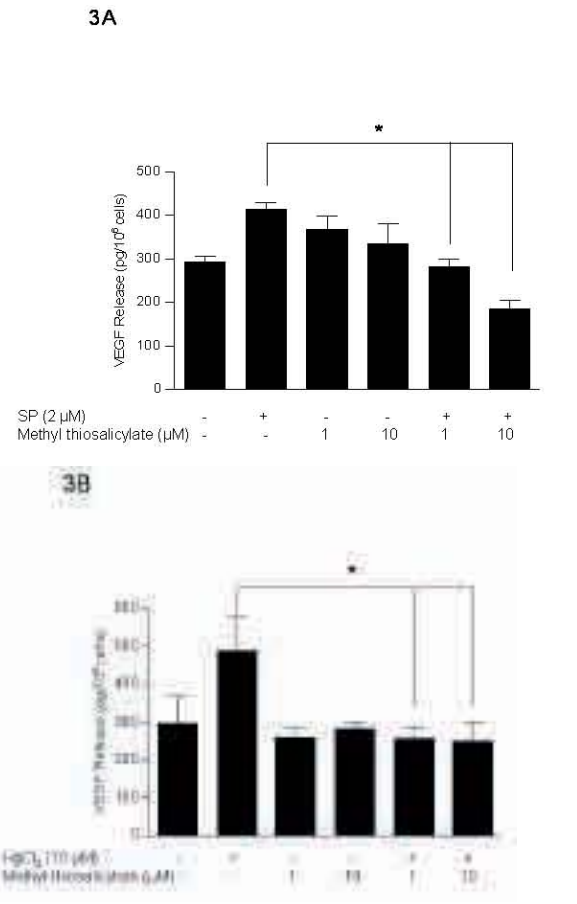

Fig 3. *Effect of methyl thiosalicylate on (**A**) SP and (**B**) $HgCl_2$-induced VEGF release. Mast cells were incubated with methyl thiosalicylate and SP or $HgCl_2$ for 24 h at 37°C. The cells were then centrifuged and the supernatant fluid was collected. VEGF release was assayed by ELISA (n=3, p<0.05).*

µM (326±12pg/10^6 cells, 335±12 pg/10^6 cells), respectively, compared to control (242.5±21 pg/10^6 cells) (n=5, p<0.05, Fig. 1B).

Effect of thimerosal on SP-induced VEGF release

$HgCl_2$ (10 µM) added together with SP (2 µM) had a statistically significant effect in augmenting VEGF release (553±63 pg/10^6 cells) as compared to SP alone (445±16 pg/10^6 cells) (Fig. 2A). In contrast, thimerosal (10 µM) added together with SP (2 µM) inhibited SP-induced VEGF release to 274.5±35 pg/10^6 cells (n=3, p<0.05, Fig. 2B). There was no statistical difference between thimerosal alone and thimerosal with SP.

Effect of methyl thiosalicylate on $HgCl_2$ and SP-induced VEGF release

In view of the fact that thimerosal inhibited the effect of SP, we hypothesized that the inhibition was due to its thiosalicylate component. We therefore examined the effect of methyl thiosalicylate on SP-induced VEGF release. Methyl thiosalicylate (1, 10 µM) added with the trigger inhibited SP (2 µM)-induced VEGF release from 413.72±18 pg/10^6 cells to 281.63±24 pg/10^6 cells at 1 µM and 185.85±23 pg/10^6 cells at 10 µM (Fig. 3A). Methyl thiosalicylate

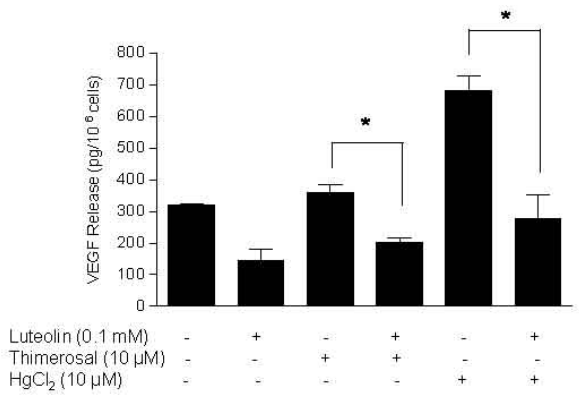

Fig 4. *Effect of pretreatment with luteolin on thimerosal and $HgCl_2$-induced VEGF release. VEGF release was assayed by ELISA (n=3, p<0.05).*

(1, 10 µM) also inhibited $HgCl_2$ (10 µM)-induced VEGF release from 488±76 pg/10^6 cells to 258±29 and 249.5±24 pg/10^6 cells, respectively (n=3, p<0.05, Fig. 3B).

Effect of luteolin on thimerosal and $HgCl_2$-induced VEGF release

Luteolin is a flavonoid known to inhibit mast cell secretion. Therefore, we examined whether luteolin could inhibit thimerosal - or $HgCl_2$-induced VEGF release. Pretreatment (10 min) of LAD2 cells with luteolin (0.1 mM) blocked VEGF release (100% inhibition) stimulated by either thimerosal (10 µM) or $HgCl_2$ (10 µM) (n=3, p<0.05, Fig. 4).

DISCUSSION

This is the first report to our knowledge showing that luteolin and methyl thiosalicylate can inhibit SP and $HgCl_2$-induced VEGF release from human cultured mast cells. The mechanism of thiosalicylate inhibition is not clear. Sodium salicylate and ibuprofen had no effect at the same concentration as thiosalicylate, implying that cyclooxygenase is not involved. Luteolin and thiosalicylate may work by preventing intracellular calcium elevations since thimerosal increased cytosolic calcium levels in thymus lymphocytes (4) as $HgCl_2$ did in PC12 cells (2).

Mast cells are potential targets for environmental agents with immunotoxic effects because they are mostly located in the skin, respiratory and gastrointestinal tracts (5). Mast cells are critical for allergic reactions, for innate and acquired immunity (6), as well as in inflammation (7). Non-allergic mast cell triggers can derive from either the gut or the brain (7), and include neuropeptides such as SP (8). Once activated, mast cells secrete numerous vasoactive, pro-inflammatory and neurotoxic molecules; these include histamine, prostaglandin D_2, IL-6 (7) and VEGF (9-10), an isoform of which is vasodilatory and is overexpressed in delayed hypersensitivity reactions (11). In fact, mast cells can release VEGF (12), IL-6 (13) and other mediators "selectively" without degranulation (14), leading to "allergic-like" symptoms not evidenced by typical diagnostic tests. Such mediators could disrupt the blood-brain barrier (BBB) (15-16) permitting brain inflammation and further increasing brain $HgCl_2$ levels, especially since $HgCl_2$ can cross the BBB through a transport mechanism (1, 17).

Methyl mercury ingestion from fish was previously linked to neurological damage (18). Exposure to mercury at critical developmental periods (1, 19) may contribute to the pathogenesis of neurodevelopmental disorders such as autism, especially in subjects with autism susceptibility genes (19). Such subjects may be further vulnerable because of mast cell sensitivity or activation by other triggers (20). For instance, a preliminary report indicated that the incidence of autism is 10-fold higher in mastocytosis patients (1/10 children) (21), characterized by increased number of hyperactive mast cells in the skin and other tissues (22), than the general population (1/100 children) (23).

The ability of the natural flavone luteolin (24) to inhibit the effect of SP and $HgCl_2$ on VEGF release is impressive. Luteolin can inhibit mast cell activation and mast cell-dependent stimulation of activated T cells (25). Luteolin is anti-inflammatory (26), inhibits IL-6 release from microglia (27), and can inhibit autism-like behavior in mice (28). Luteolin (5, 7, 3', 4'-tetrahydroxyflavone) is closely related to 7, 8-dihydroxyflavone recently shown to mimic brain-derived neurotrophic factor (BDNF), which is neuroprotective (29). Luteolin could, therefore, be useful in treating neuroinflammatory diseases either alone or as an adjuvant to other therapeutic approaches (30).

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