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Research Article

Effects of phytoalexin on human leukocyte release, hormone, chemical, and mast cell proliferation.

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Abstract

Rich in red wine, peanuts, and grape skin, resveratrol is a polyphenol that has been demonstrated to possess anti-inflammatory, anti-cancer, and antioxidant properties. It may help mitigate allergic inflammation. We looked at how resveratrol and the anti-allergy medication tranilast affected human mast cell activation. Resveratrol and tranilast both prevented substance P, IgE/anti-IgE, and compound 48/80 from inducing degranulation in LAD2 mast cells. When added concurrently to physiological stimuli, resveratrol inhibited degranulation immediately and maintained the effect for up to 24 hours. Although cAMP was not necessary for the inhibitory effect, it might have been caused by calcium modulation, resveratrol, and, to a lesser extent, tranilast, which preceded substance P-induced elevations.

While tranilast had no effect, resveratrol reduced substance P-induced TNF and MCP-1 production as well as the IgE-mediated release of cysteinyl leukotrienes. Moreover, on LAD2 cells, resveratrol and tranilast decreased the production of FcεRI, a high affinity IgE receptor. Human primary CD34+-derived mast cells (HuMC) showed a greater response to resveratrol's effects on mast cell activation, and the polyphenol was found to be substantially more effective in these cells than tranilast. In summary, resveratrol was as effective as, if not more so than, the anti-allergy medication tranilast in inhibiting important elements of human mast cell activation in response to physiological stimuli. Resveratrol may therefore be a useful medicinal substance for the management of allergic illness.

Keywords : *Mast Cells; Resveratrol; Tranilast; IgE;Substance P; Leukotrienes*

Introduction

Certain plants respond to infections by producing resveratrol, an antibacterial substance [1,2]. Red wine, peanuts, and grape skins are the foods highest in polyphenols. This has prompted much investigation into the possible health advantages of red wine, while it is questionable if the resveratrol concentration is sufficient for bioactivity. Numerous health-promoting properties of the polyphenol have been linked to it, most of which have been observed in mice. These properties include the prevention of cancer, antioxidant and anti-inflammatory activity, cardioprotective effects, and dilatation of the airways [3, 4]. As resveratrol inhibits the release of arachidonic acid and the synthesis of lipoxygenase and cyclooxygenase products [5-7], it may also be advantageous for allergic inflammatory reactions.

Key players in the mediation of allergy and anaphylactic responses, mast cells release pro-inflammatory mediators when allergens crosslink IgE coupled to FcεRI [8,9]. In an OVA-induced mouse asthma model, resveratrol decreases airway hyperresponsiveness, inflammation, and allergen-specific IgE levels in vivo [10]. Resveratrol-induced inhibition of degranulation and histamine release in mouse bone marrow mast cells (BMMC) [11], rat basophil-like cells (RBL-2H3) [12,13], and rat peritoneal mast cells [14] has been observed in preliminary in vitro animal studies. This inhibition is most likely caused by suppressing tyrosine phosphorylation of ERK and PLC 1 [12]. Additionally, resveratrol lowers the synthesis of prostaglandin D2, TNF, and cysteine-teinyl leukotriene in mouse BMMC [11]. There aren't many studies looking at how resveratrol affects human mast cells.

Determining the impact of resveratrol on critical elements of human mast cell activation to physiological stimuli, such as degranulation and inflammatory mediator release, was the aim of this investigation. We used LAD2 mast cells and CD34+ cell-de-

rived human primary mast cells (HuMC) to achieve this. Additionally, we contrasted resveratrol's effects with those of tranilast, an anti-allergy drug now on the market.

MATERIAL AND METHODS

Human Mast Cell Growth

The LAD2 cells were cultivated in serum-free media (StemPro-34 SFM, Life Technologies, Burlington, ON) supplemented with 100 ng/ml of stem cell factor (Peprotech Inc., Rocky Hill, NJ), 100 U/ml of penicillin, and 50 µg/ml of streptomycin. The expression of kit and FcεRI was periodically assessed in LAD2 cells using flow cytometry, and the results showed that the expression of these receptors was comparable to that of CD34+ cells obtained from blood. In StemPro-34 SFM supplemented with 2 mM L-glutamine, 50 µg/ml streptomycin, 100 IU/ml penicillin, 100 ng/ml SCF, and 100 ng/ml recombinant human IL-6 and recombinant human IL-3 (PeproTech) (30 ng/ml; first week only), human peripheral blood-derived CD34+ cells were grown. Both LAD2 and HuMC were grown in serum-free medium (StemPro-34 SFM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml SCF for investigations that required overnight incubation.

Assay for Degranulation

After being suspended in buffer, cells were stimulated for 30 minutes at 37˚C/5% CO2 using either 0.3 µg/ml compound 48/80 or 0.5 µg/ml substance P (Sigma-Aldrich Canada, Oakville, ON). For IgE assays, cells were sensitised with 0.5 µg/mL myeloma IgE (Calbio-chem, Billerica, MA) overnight at 37˚C/5% CO2, then stimulated for 30 minutes with 100 µg/ml rabbit anti-IgE (Dako, Carpinteria, CA) or 0.1 µg/ml IgE-biotin (Abbiotec, San Diego, CA) overnight at 37˚C/5% CO2, and 30 minutes with 0.5 µg/ml streptavidin.

Before being stimulated by an agonist, cells were pretreated for the given amounts of time with resveratrol or tranilast (Sigma-Aldrich). After hydrolyzing sub-stratum p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 90 minutes at 37˚C, the amounts of β-hexosaminidase in the supernatants and cell lysates were measured. The β-hexosaminidase release percentage was computed as a percentage of the overall content.

Imaging of Calcium

After loading cells with 1 µM fura-2 AM (Invitrogen, USA) in HEPES buffer for 30 minutes at 37˚C, they were washed and incubated for another 15 minutes at 37˚C in BSA-free Hepes. Under an inverted fluorescent microscope, cells were positioned on a poly-D lysine-coated glass bottom dish and stimulated at 340 and 380 nm using a high-speed filter changer.

Using SlideBook for Stallion, version 4.26.04 software (Intelligent Imaging Innovations, USA), fluorescence measurements and calcium responses were recorded at 100 msec intervals. Following the establishment of the basal calcium signal, resveratrol, tranilast (10 µg/ml), and substance P (0.5 ug/ml) were administered. Twenty randomly chosen cells' calcium responses were examined for each condition (and again for n = 3) and plotted as the ratio of 340 to 380 as a function of time.

ELISA Analysis of MCP-1 and TNF

After a three-hour pretreatment at 37˚C with 5% CO2, the cells were stimulated for twenty hours with 0.5 µg/ml substance P. Centrifugation was used to separate the cell-free supernatants, and commercial TNF and MCP-1 ELISA kits (eBiosciences, San Diego, CA) were used to measure human cytokine expression.

Cysteinyl Leukotriene EIA Analysis

The cells were treated with resveratrol or tranilast for three hours at 37˚C and 5% CO2 after being sensitised with 0.5 µg/ml IgE for the previous night. After that, cells were stimulated with 100 µg/ml of anti-IgE. Cysteinyl leukotriene (cysLT) levels were assessed using commercial competitive enzyme immunoassays (Assay Designs) after cell free supernatants were separated by centrifugation.

CAMP EIA Analysis

After treating the cells for 30 minutes with 10 µg/ml resveratrol, tranilast, or 10 µM forskolin, the process was halted by adding 5 M HCL and waiting 20 minutes before using a pipette to triturate the mixture. Centrifugation was used to extract cell-free supernatants, and a commercial competitive immunoassay (Cayman Chemicals, Ann Arbour, MI) was utilised to quantify cAMP.

PCR in Real Time

After a three-hour pretreatment with either resveratrol or tranilast, the cells were stimulated for three hours at 37˚C/5% CO2 with 0.5 µg/ml of Substance P. Using the Tri Reagent technique, total RNA was extracted from each treatment (Sigma-Aldrich Canada, Oakville, ON). Using oligo dT primer (IDT; Integrated DNA Technologies) and M-MLV reverse transcriptase enzyme

(Invitrogen, Burlington, ON), 1µg of total cellular RNA was reverse transcribed to create cDNA. Quantitative PCR was used using a Step One Plus system (Applied Biosystems, Foster City, CA) to analyse gene expression. Each quantitative PCR test employed 20 ng of cDNA, and each reaction was run in duplicate for 40 cycles.

Flow Cytometry-Based Expression of FcεRI

For 20 hours at 37˚C and 5% CO2, cells were treated with resveratrol or tranilast. After being cleaned and resuspended in 0.1% BSA/PBS, they were kept on ice for ten minutes. After that, the cells were treated for one hour at 4˚C with PE-conjugated anti-FcεRI antibody or isotype control antibody (eBiosciences, San Diego, CA). The cells underwent two rounds of washing in 0.1% BSA/PBS, were resuspended in the same buffer, and then examined using a BD Biosci- ences, Mississauga, ON, FACSArray flow cytometer. The expression for FcεRI levels is mean fluorescence intensity (MFI).

Examination of Statistical Data

The means ± standard errors were used to describe the results. One-way ANOVA or the Student's t test were used to analyse the data.

for every experimental condition, with a minimum of three repetitions of each experiment, and data analysis using a repeated measures ANOVA combined with the Tukey Post Hoc test. Using GraphPad Prism software (GraphPad Software Inc., San Diego, CA), non-linear regression and 4-parametric analysis were used to calculate the IC50 values. After accounting for the effects of the vehicle (DMSO) on all data, a P value of less than 0.05 was considered significant.

RESULTS

Inhibition of Degranulation Mast Cells

LAD2 cells were pre-treated with varying concentrations of resveratrol and tranilast for 30 minutes before being stimulated with either substance P (0.5 µg/ml), compound 48/80 (0.3 µg/ml), or IgE/anti-IgE (0.5 µg/ml/100 ng/ml) (Figure 1). This was done to ascertain the impact of the two compounds on human mast cell degranulation. The degranulation caused by substance P (68% and 52%, respectively), compound 48/80 (80% and 51%), and IgE/anti-IgE (37% and 27%, respectively) was suppressed by both resveratrol and tranilast (100 µg/ml).

Immediate Effects on Degranulation

In order to ascertain the ideal resveratrol inhibitory effects, multiple treatment intervals were examined. Before being activated with 0.5 µg/ml substance P, 0.3 µg/ml compound 48/80, or 0.5 µg/ml streptavidin (in 0.1 µg/ml IgE-biotin sensitised cells), LAD2 cells were pretreated with resveratrol for 0, 30, 3, and 24 hours. At every time point examined, resveratrol dramatically reduced substance P- and IgE-mediated human mast cell degranulation (Figures 2(a) and (c), respectively). Additionally, compound 48/80-induced degranulation was blocked by resveratrol, albeit less successfully and at earlier times needing greater doses to block reactions. IC50 values varied from 1.34 to 12.5 ug/ml according on the stimulant used and length of resveratrol treatment, as determined by centration response curves as shown in Figure 2(d).

Calcium Intracellular Regulation

Since degranulation depends on calcium, we looked at how 10 µg/ml resveratrol and tranilast affected intracellular calcium levels when added concurrently with substance P. In LAD2 cells, resveratrol and tranilast reduced substance P-dependent increases in intracellular calcium by 98% ± 15% and 45% ± 36%, respectively.

No Impact on the Production of cAMP

We investigated the effects of resveratrol andtranilast on cAMP synthesis in LAD2 cells in contrast to the adenylate cyclase activator, forskolin, because raising cAMP can decrease mast cell degranula-tion. At 10 µg/ml, neither substance affected the production of cAMP, but the addition of 10 µM forskolin 3.5 doubles the levels of cAMP.Blocking the Production of Cytokines and Chemokines It has been demonstrated before that substance P causes human mast cells to release chemokines and cytokines. Therefore, it was determined if resveratrol and tranilast could stop substance P from causing MCP-1 and TNF to be produced in LAD2 cells. After three hours of pretreatment, TNF production was decreased by both resveratrol and tranilast (Figure 4(a)). Compared to tranilast (35% ± 6%; p = 0.06), resveratrol was shown to be a more powerful inhibitor, lowering TNF release by 61% ± 2% at 10 µg/ml. There was neither additive nor synergistic impact from the two compounds together.

30 minutes of pre-treatment had no discernible impact on substance P-induced TNF release, suggesting that shorter incubations with the compounds were insufficient to reduce TNF production (data not shown). After three hours of pretreatment

with 20 µg/ml resveratrol, TNF mRNA expression was decreased 51% ± 12%, but no effect was seen. On the other hand, MCP-1 mRNA expression was completely prevented with 100 µg/ml resveratrol (Figure 4(d)). On MCP-1 expression or production, trenillast had no discernible impact, and the combination of the two substances had no further effects.

Prevention of the Production of Cysteinyl Leukotrienes

After three hours of pretreatment, IgE-sensitized LAD2 cells produced 27%± 14% less cysteinyl leukotrienes when exposed to 10 µg/ml resveratrol than when not treated with anti-IgE; however, this difference was not statistically significant.

FcεRI Expression Downregulation

Mast cell activation is mediated by IgE through its high affinity receptor, FcεRI. Therefore, following a 20-hour treatment, we examined the impact of resveratrol and tranilast on the expression of FcεRI in LAD2 cells. Resveratrol was more effective than tranilast, reducing baseline FcεRI expression by 28% ± 0.4% as opposed to 14% ± 2% by tranilast, even though both drugs significantly reduced FcεRI expression at 100 µg/ml (Figure 4(f)).

Impact on HuMC Cellular

Finally, we looked at how tranilast and resveratrol affected primary human mast cells, or HuMC. When compared to LAD2 cells, the effects of resveratrol were more pronounced in HuMC. Resveratrol pretreatment for thirty minutes led to a considerable reduction in IgE/anti-IgE-induced degranulation of HuMCs, with a 62% ± 7% inhibition at 10 µg/ml. Tranilast did not significantly decrease HuMC degranulation, in contrast to LAD2 cells (Figure 5(a)). Similarly, Figure 5(b) shows that a 3-hour pretreatment with 10 µg/ml resveratrol almost completely eliminated IgE/anti-IgE-induced cysteinyl leukotriene synthesis by 90% ± 1%. Similar to LAD2, tranilast had no impact on the generation of cysteinyl leukotrienes. The combination of resveratrol and tranilast had no further effects.

DISCUSSION

The primary mediators of allergic inflammation and anaphylactic reactions are mast cells, which release pro-inflammatory cytokines and chemokines along with histamine, proteases, and metabolites of arachidonic acid in response to various stimuli. These ubiquitous cells are found throughout vascularized tissue, especially at sites of pathogen entry. Neuropeptides like substance P, complement proteins, elements of bacteria, viruses, and parasites, as well as the antigen-crosslinking of IgE bound to its high affinity receptor FcεRI, can all activate these ubiquitous cells.

Resveratrol, a polyphenol, has been demonstrated in several studies to have inhibitory effects on murine mast cell lines. According to Kang et al., resveratrol prevents the human mast cell line HMC-1 from producing chemokines when non-physiological stimuli like PMA and calcium ionophore are applied. This work was further hampered by not examining degranulation or the release of metabolites derived from arachidonic acid, in addition to the cells' incapacity to respond to IgE [15]. Here, we report the first investigation of resveratrol's effects on human mast cell activation to physiological stimuli utilising primary mast cells obtained from peripheral blood and IgE-responsive LAD2 cells, which resemble primary mast cells more than HMC-1. We discovered that resveratrol, perhaps by controlling calcium flux, decreased all the key elements of human mast cell activation, such as degranulation and the production of pro-inflammatory cytokines, chemokines, and cysteinyl leukotrienes in LAD2 cells.

Resveratrol exhibited more pronounced effects on degranulation and cysteinyl leukotriene release in human primary mast cells (HuMC), and in both cell types, the polyphenol demonstrated much greater efficacy than the commercial anti-allergy medication tranilast.

Resveratrol has a low bioavailability; in humans, it is metabolised two to four hours after oral dose and within thirty minutes after intravenous treatment [16, 17]. Because of the compound's brief half-life, effects on mast cell activation would need to happen quickly. Here, we demonstrate that when resveratrol is given concurrently with physiological stimuli, it inhibits degranulation and calcium influx immediately and has a 24-hour duration of action. Extended (3 hours) resveratrol treatments were necessary to impede slower Therefore, resveratrol may be able to prevent mast cell activation and lessen allergic inflammation if consumed in sufficient amounts. Moreover, resveratrol metabolites may possibly be physiologically active since they are more stable and bioavailable than their parent substance (half life of 7–14 hours in serum) [3,16]. It appears that resveratrol is not influencing degranulation responses via a complicated signalling mechanism based on its immediate effects. Instead, it's possible that resveratrol is attaching itself to the stimuli in order to block the cell from interacting with it. This seems improbable, though, given the stark structural variations among the three

stimuli—one is a tiny peptide, the other is a big immunoglobulin protein, and the third is a chemical molecule. Furthermore, one would anticipate that all mast cells would be prevented from interacting with stimuli.

Contrary to expectations, resveratrol significantly decreased IgE-mediated degranulation by up to 81% while having no effect on IgE-mediated cysteinyl leukotriene synthesis. Furthermore, compared to degranulation, resveratrol required far longer treatment periods (3 hours) to have any effect on cytokine responses and was differentially effective in its ability to decrease the production of TNF and MCP-1. It is more likely that resveratrol works via inhibiting calcium signalling. The increase in calcium levels in the cytosol after cellular activation is essential for mast cell degranulation [18, 19]. When substance P was administered concurrently with 10 µg/ml resveratrol, we observed that the calcium flow was effectively eliminated. In the same experimental setup, this resveratrol concentration resulted in a 53% suppression of degranulation.

The quickness of this reaction implies that resveratrol is preventing calcium flow immediately, perhaps through blocking calcium channel activation directly. Similar partial reductions in PMA/calcium ionophor-induced calcium flux were also shown by Kang et al. in HMC-1 mast cells, however they did not associate this with degranulation responses [15].

Changes in cyclic nucleotides can also affect degranulation. For example, cAMP elevating agents like PGE2 [24], methanan-damide [22], and β2 adrenergic agonists [23], as well as cAMP analogues [20], cAMP phosphodiesterase inhibitors [21], and the AC cyclase activator forskolin [22, 23], all inhibit mast cell degranulation. However, we did not see an increase in cAMP levels after resveratrol therapy, indicating that cAMP is not the cause of the polyphenol's inhibitory actions.

Resveratrol regulation of transcription factor activation may be responsible for the study's observed inhibition of MCP-1 and TNF production. Indeed, in several cell types, resveratrol is shown to decrease NF-κB transcription factor activation and nuclear translocation [15, 25, 26]. Moreover, resveratrol prevents U-937 histiocytic lymphoma cells from activating the AP-1 transcription factor [25]. Resveratrol inhibits the activation of IκB kinase and subsequent IκB phosphorylation in THP-1 monocytes [26], but not in U-937 histiocytic lymphoma cells [25], suggesting that this inhibition is likely mediated upstream of the transcription factors. Furthermore, Kang and colleagues documented a decrease in ERK activation and IκB degradation in HMC-1 cells, potentially explaining the lowered production of TNF, IL-6, and IL-8 in these cells [15].

Conclusion

Ultimately, our findings demonstrate that resveratrol suppresses the main mechanisms of mast cell activation, in part by reducing calcium flux, and that these actions took place within the range of bioavailability that has been documented. Furthermore, resveratrol outperformed the over-the-counter allergy medication tranilast as an inhibitor of mast cell activation. As a result, resveratrol might be a strong contender for an alternative allergy inflammation treatment.

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