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Research Article

THE PATHOLOGICAL EFFECTS OF RESVERATROL ON NEUTROPHIL ACTIVATION AND INFLAMMATION: IN VITRO FINDINGS

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ABSTRACT

Resveratrol (RES), a polyphenolic compound found in various plants, has demonstrated significant antioxidant and anti-inflammatory properties. This study investigates the pathological effects of RES on neutrophil activation and inflammation in vitro. Neutrophils, as key players in immune responses, produce reactive oxygen species (ROS) during inflammatory processes, contributing to tissue damage in various diseases. RES was tested on human neutrophils in both intracellular and extracellular settings to evaluate its ability to modulate oxidative burst and neutrophil activity. In this study, RES was found to dose-dependently inhibit neutrophil chemiluminescence (CL) induced by phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187. RES treatment reduced the oxidative burst, as well as the production of free radicals, in both whole blood and isolated neutrophils. Furthermore, RES inhibited the phosphorylation of protein kinase C (PKC) isoenzymes α and βII, which are involved in the activation of neutrophils and subsequent ROS production. Although RES did not induce significant apoptosis in neutrophils, it effectively reduced nitrite accumulation in macrophages, suggesting a suppression of inducible nitric oxide synthase (iNOS) activity. These findings indicate that RES interferes with key signaling pathways in neutrophils, reducing oxidative stress and inflammatory responses. RES may provide therapeutic potential for treating inflammatory disorders such as ischemic reperfusion injury and other conditions involving excessive neutrophil activation.

Keywords: - Resveratrol, Neutrophil Activation, Oxidative Burst, Inflammation

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INTRODUCTION

In areas of inflammation, neutrophils produce oxygen radicals. Antimicrobial and tumoricidal ROS are produced during inflammatory environments, which are often referred to as "oxidative bursts" and provide protection against pathogens in the environment. It is also interesting to note that neutrophils can lead to tissue-damaging inflammatory reactions, which underlie many inflammatory conditions. [1] A number of signaling pathways are involved in triggering neutrophil activation. One involves the protein kinase C pathway, which is triggered by pentamyristate (PA), in addition to the Src family of protein tyrosine kinases. Whether neutrophils are in circulation or emigrated depends on whether they are going through an apoptosis process. Inflammation is caused by neutrophil apoptosis, which may be a therapeutic target in the future. [2 - 4] Consequently,

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neutrophils do not apoptose in a timely manner, which causes inflammation to prolong, worsen, or, in extreme cases, prevent spontaneous resolution of inflammation. The neutrophils promote apoptosis, which reduces inflammation [5, 6]. A wide range of phenolic substances, including those found in edible and medicinal plants, have been reported to modulate important cellular signaling processes to combat oxidative damage, carcinogenesis, and mutation. Natural polyphenols enhanced the apoptosis of stimulated neutrophils and decreased the activity of protein kinase C thereby suppressing oxidative burst [7]. It has been shown that RES, which is a polyphenolic phytoalexin, provides a wide range of biological and clinical benefits [8]. In addition to antioxidant and antiinflammatory properties, RES has been shown to have antiproliferative, antiangiogenic, and anti-inflammatory properties [9]. Resveratrol is mentioned in nearly 5000 articles in PubMed, but molecular research to determine its mechanism has not been conducted. An assessment of RES was conducted in whole blood, neutrophils isolated intracellularly and extracellularly, protein kinase C activation, caspase-3 activity, and cell viability in this study. In cell-free systems, RES also scavenges free radicals, absorbs oxygen radicals, prevents hydroxyl radical formation, scavenges ROS, inhibits lipid peroxidation, and produces nitricoxide.

MATERIAL AND METHODS

In addition to the compounds listed above, Sigma-Aldrich also offered tetramethylchroman-2-carboxylic acid (Trolox), fluorescein dihydrochloride, and cobalt fluoride hexahydrate. The companies that supplied picolinic acid included Fluka and Enzo Life Sciences. [10] All chemicals used, except for these, were analytical grade and obtained from reputable commercial sources. The patient, who was between the ages of 20 and 50, was treated for anemia during venous puncture. Trisodium citrate was used as an anticoagulant in the treatment of blood clots. Blood sampling was authorized for National Transfusion Service under NTS-KRA/2012/SVI. Scientists have successfully isolated neutrophils from whole blood in the past.

The viability of neutrophils was increased by more than 96% when trypan blue exclusion and control chemiluminescence were used. Human whole blood was diluted with a citrate solution, as described above. Dextran (3%) was added as an additive to the mixture and centrifuged at 10 g for 10 minutes at room temperature. Leukocytes were collected from buffy coat by collecting approximately 1 mL and storing it on ice for 24 hours. Granulocytes were counted using a Coulter Counter. [11] With the suspension adjusted, 105 neutrophils were obtained. An incubation at 37°C was conducted with three different concentrations of RES and a control sample for 10 minutes. stained cells with annexin V for 10 minutes, then applied propidium iodide (1 g/mL), and analyzed them immediately with a cytometer. As said above, phosphorylation of PKC isoenzymes α and βII has been observed [12].

Incubation with RES, stimulation with PMA (0.15 mM, one minute), and lysation with solubilization buffer were performed on 5 106 human neutrophils. By sonicating on ice, centrifuging, diluting the supernatants, and then adding the buffer for five minutes, 9.8% polyacrylamide gels were loaded. After 60 minutes of blocking with 1% bovine serum albumin, a polyacrylamide gel with 9.8% concentration was loaded. Antiphospho-PKC I and II antibodies are incubated with rabbit anti-human antibodies, 1:8000, Cell Signaling Technology, Danvers, as a matter of routine. Approximately 60 minutes after six washes with TBS, rabbit anti-rabbit antibodies were incubated. Each PKC band's optical density was adjusted by using its respective β-actin band. ATC RAW 264.7 macrophages were cultivated on 10% fetal bovine serum and 1% gentamycin-containing Dulbecco's Modified Eagle Medium (Sigma, USA). [13]

Optimal cell growth was achieved by maintaining a 37°C, 5% CO2 environment. Harvested cells are washed after reaching confluence. A ATP test determined the number of cells and their viability. The supernatants of murine macrophages were measured using a nitrite assay. There was no response to a compound in cells treated with LPS but not with RES. 5000 g centrifuges were used to centrifuge the incubated media after 5 minutes at 4°C. [14] The supernatant from each sample was mixed with Griess reagent during the overnight dark period. In these samples, NO synthase expression is elevated via Western blot analysis. Following removal of the supernatant, nitrite measurements were performed, followed by application of the lysis buffer (1% sodium dodecyl sulfate, 0.1 M Tris pH 7.4, 10% glycerol, 3.0 M sodium orthovanadate, 3.0 M phenylmethanesulfonyl fluoride) to remaining cells. We measured protein concentrations using the Pierce BCATM protein assay. SDS-polyacrylamide was used to electrophorese an equal amount of protein on a 7.5% polyacrylamide gel. It was determined that iNOS protein expression is quantified using Western blot analysis [15]. The density values of each band were expressed using arbitrary units using Image JTM to quantify relative protein amounts.

Electrochemical tests were performed to see how well extracts scavenged NO in chemical systems. Porphyrin microscopic electrodes, counter electrodes, and reference electrodes were used to measure NO. The measurement of NO was performed with potentiometers. [16] A measurement vial filled with NO-saturated water leads to a rapid increase in the induced signal, which

gradually declines until its background level is reached. Following application of the extracts, we measured how long it took for the background current to reach its previous level. [17]

To each 0.1 mL sample, 0.5 mM beta-linolenic acid was added. In order to induce lipid peroxidation, Co(II) and hydrogen peroxide were added as a system that generates hydroxyl radicals (see section HORAC for details). After 2 hours of incubation, the mixture was cooled to room temperature.

Statistical Analysis

As a general rule, data are represented as means x standard deviations, unless otherwise noted. This study utilized the ANOVA paired test for comparison between the treatment and control groups. Statistical significance was determined when P = 0.05 or P = 0.01 distinguished two groups.

RESULTS

The Rank-Order Efficacy Experiment demonstrated that RES significantly inhibited CL when compared with the potencies of PMA, fMLP, OPZ and A23187, showing that resveratrol has a different ability to reduce stimulated chemiluminescence. Neutrophils with intact membranes contain 3.8 nM ATP (3.2% of total ATP). Due to RES's inability to release ATP (results not shown), it cannot disintegrate isolated neutrophils. When administered at concentrations between 1 and 100 mM, RES reduces neutrophil viability. [18] A significant difference in cell death was not observed between cells treated with RES at 1 and 10+M and control cells. In contrast to controls, RES concentrations significantly reduced the number of viable cells from 7.9% to 18.6%. There was no increase in the number of dead cells. This study demonstrated that the optimal concentration of RES (1 and 10 mM) did not affect neutrophil apoptosis.

Table 1: The viability of neutrophils is affected when resveratrol is contaminating human blood cells at levels of 1 to 100 mM. The cells were treated with annexin V-FITC conjugate of resveratrol (40 mM), stimulated with resveratrol for 10 minutes, stained with propidium iodide (1g/mL), and analyzed on a Beckman Coulter Cy immediately. Averaging four to six participants.

Resveratrol (μM)	Live cells	Apoptotic cells	Dead cells
0	45.45 ± 1.02	4.45 ± 2.00	0.10 ± 0.02
1	47.20 ± 1.15	4.30 ± 2.15	0.10 ± 0.03
10	42.05 ± 2.08	2.20 ± 2.30	0.05 ± 0.01
100	41.10 ± 1.33	09.15 ± 2.70	0.10 ± 0.01

DISCUSSION

A number of polyphenols, including curcumin, pterostilbene, pinosylvin, and N-feruloyl serotonin, have been shown to reduce neutrophil ROS production. Chemiluminescence activated by fMLP does not result in the production of leukotriene B4, 6-trans-LTB4, or 12-trans-epi-LTB4. [19] Multiple studies have shown that transresveratrol inhibits polymorphonuclear leukocyte proliferation. A study in isolated neutrophils showed that RES prevented disintegration by preventing ATP release. Researchers have also shown that RES reduces spontaneous ATP synthesis in a dose-dependent manner. Although the concentration of dead cells was highest, the percentage of live cells decreased by 10.7% (Table 1). As a result of treatment with 10-mM RES, the caspase-3 activity in cell-free systems was significantly reduced. Apoptosis occurs when cells are treated with RES in the cellular model, which kills them. The reduction of intracellular reactive oxygen species, increased autophagy in human U251 glioma cells, cleaved caspase-8 and caspase-3 in colon cancer cells, and induced apoptosis in chronic myeloid leukemia cells is also supported by RES. Tests were conducted to evaluate the antioxidant properties of RES, including the ORAC (peroxyl) and HORAC (hydroxyl) tests, chemiluminescence tests, NO scavenging, and lipid peroxidation inhibition tests. [20] Our chosen methods can be integrated to investigate various aspects of antioxidant action, allowing us to determine the sample's overall antioxidant potential. The NO scavenging properties of RES were also evaluated using electrochemical analysis. At any concentration of resveratrol, NO was not scavenged. [21] RES increases antioxidant enzyme activity through this mechanism, suggesting that it is a potent antioxidant and free radical scavenger. Statins are dose-dependent because they induce lipid peroxidation and atherosclerosis due to hypercholesterolemia.

Neutrophils stimulated with PMA exhibited reduced protein kinase C activation, indicating that RES interfered with neutrophil oxidative burst.[22] Human gastric adenocarcinomas and CaSki cellsserotonin, as well as the polyphenol N-feruloyl serotonin in neutrophils. By inhibiting PKC activation RES may alter intracellular signalling pathways that regulate COX-2 expression and iNOS function, as well as NF-κB activation. Biological and microbiological processes are controlled by nitric oxide, which is a member of reactive

nitrogen species. [23] LPS-stimulated macrophages produced little NO in the presence of RES. It is consistent with the latest results obtained by other researchers who have also found that RES administration suppresses inducible nitric oxide synthase activity and NO production in macrophages. [24-25] The mechanism by which resveratrol reduced nitrite accumulation in stimulated macrophages is less dependent on the regulation of iNOS expression than on nitrite reduction. Electrochemical measurements, however, showed that RES could not scavenge NO, suggesting that RES can be excluded from having a direct scavenging effect on NO due to its inhibitory action. A chemiluminescence measurement showed that RES can inhibit oxidative bursts produced by stimulated blood and neutrophils as well as those generated by cell-free systems. A cell-free system using RES has been shown to inhibit oxidative bursts in human blood and neutrophils, suppress free radicals.

CONCLUSION

This study provides valuable insights into the pathological effects of resveratrol (RES) on neutrophil activation and inflammation, highlighting its potential as a therapeutic agent in managing inflammatory diseases. The in vitro findings indicate that RES exerts a significant influence on neutrophil function by modulating oxidative stress and inflammatory pathways, key contributors to tissue damage in various pathological conditions. Neutrophils are pivotal in the body's immune response, particularly in the production of reactive oxygen species (ROS) during inflammatory events. While ROS play a critical role in pathogen defense, their excessive production can lead to tissue injury, contributing to chronic inflammation and disease progression. RES has been shown to reduce the oxidative burst in neutrophils, which is typically induced by stimuli like PMA and the calcium ionophore A23187. By inhibiting ROS production, RES mitigates the harmful effects of neutrophil activation, which could be beneficial in conditions like ischemic reperfusion injury, rheumatoid arthritis, and other inflammatory diseases. Furthermore, the study demonstrated that RES treatment suppressed the phosphorylation of protein kinase C (PKC) isoenzymes α and βII, which are essential for neutrophil activation and subsequent ROS production. This finding suggests that RES may interfere with early signaling events involved in neutrophil activation, further limiting oxidative damage during inflammation. Additionally, RES exhibited inhibitory effects on inducible nitric oxide synthase (iNOS) activity in macrophages, thus reducing nitrite production, which is often associated with inflammation and tissue injury. Interestingly, while RES did not induce significant neutrophil apoptosis, it did demonstrate an ability to prevent neutrophil disintegration by inhibiting ATP release. This observation suggests that RES may act as a protective agent, preventing the premature death of neutrophils and ensuring a controlled inflammatory response. These findings support the idea that RES may provide a balancing effect on neutrophil activity, preventing excessive inflammation while preserving immune defense mechanisms. The results of this study highlight the potential of RES as an antioxidant and anti-inflammatory agent that could be applied to a wide range of diseases driven by excessive neutrophil activation and oxidative stress. RES's ability to modulate key inflammatory pathways, such as PKC activation and iNOS expression, offers promising avenues for therapeutic development. Future research should focus on exploring the clinical applicability of RES in managing inflammatory disorders and its interaction with other immune cells and inflammatory mediators. Its ability to modulate neutrophil activation, reduce ROS production, and suppress iNOS activity positions it as a potential therapeutic agent for treating inflammatory conditions where oxidative stress plays a significant role. Further in vivo studies and clinical trials are needed to fully understand its therapeutic potential and optimize its clinical use.

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