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Antiinflammatory Properties of the Muscadine Grape (*Vitis rotundifolia*)

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The muscadine grape possesses one of the highest antioxidant levels among fruits; yet, the effect of this fruit on mammalian metabolic systems has not received significant attention. To examine the antiinflammatory properties of the muscadine, grape skins were dried, pulverized, and extracted (10% w/v) with 50% ethanol. The extract was then tested in two different assays: the release of superoxide in phorbol myristate acetate-activated neutrophils and the release of cytokines [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-β), and interleukin-6 (IL-6)] by lipopolysaccharide-activated peripheral blood mononuclear cells. The release of superoxide and cytokines was inhibited by increasing concentrations of the extract. A 1:100 dilution of the extract inhibited superoxide release by approximately 60% while the release of TNF-α and IL-1β was reduced at a dilution of 1:200 by approximately 15 and 90%, respectively (all $P < 0.05$). The inhibition pattern on the release of IL-6 was similar to that seen with TNF-α. In a related in vivo study, rats were fed a diet containing 5% (wt/wt) dried muscadine grape skins for 14 days and then were injected with carrageenan in the foot pad. After 3 h, paw edema was measured and the rats on the grape skin diet had approximately 50% less paw edema than controls ($P < 0.05$). These results demonstrate that the muscadine grape skin powder possesses significant in vitro and in vivo antiinflammatory properties.

KEYWORDS: Muscadine grape (*Vitis rotundifolia*); superoxide; antiinflammatory; interleukin-1β; interleukin-6; TNF-α; edema

INTRODUCTION

Muscadine grapes (*Vitis rotundifolia*) are native to the southeastern United States and can be found growing wild from Delaware to the Gulf of Mexico and westward from Missouri to Texas. Muscadines are well-adapted to warm, humid climates in which *Vitis vinifera* do not thrive and have been grown commercially for decades. Muscadine fruits are borne in loose clusters of up to 40 grapes. Muscadine fruits are 1−1.5 in. in diameter with a thick, tough skin and several hard, oblong seeds. They are considered a southern specialty food product.

The phytochemical constituents of the whole muscadine grape differ from *V. vinifera*; muscadines have a higher total phenolic content distinguished by high ellagic, gallic, and flavonoid glycoside concentrations (*1*−*4*). The presence of ellagic acid in muscadine grapes is unique and is found in the form of free ellagic acid, ellagic acid glycosides, and ellagitannins (*1*). Another unique feature is the anthocyanin chemistries observed in muscadines. Anthocyanins are present as 3,5-diglucosides (as opposed to 3-glucosides) of delphinidin, cyanidin, petunidin, peonidin, and malvidin in nonacylated forms. These compounds and several flavonoids (quercetin, myricetin, and kaempferol) impart a dark purple color to juice and pomace. Polyphenolic anthocyanins are best known for their red and purple colors, and their antioxidant capacities have been firmly established (*5*−*7*). Anthocyanins, followed by flavonols, are the most abundant flavonoids present in whole muscadines (*1*). The major phenolics reported for the muscadine skin fraction (in descending order) are ellagic acid, myricetin, quercetin, and kaempferol while those reported for seeds are gallic acid, catechin, and epicatechin (*2*).

While muscadine and its fractions are extremely high in antioxidant activity as assessed by ORAC (oxygen radical absorbance capacity) (*1*, *4*) and FRAP (ferric reducing antioxidant potential) values (*8*), little has been documented on the effect of this fruit on biological activities and pathways in mammalian systems. In this study, the antiinflammatory properties of the muscadine grape skin are examined in several

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experimental systems. A preliminary report of these findings has been presented (*9*).

MATERIALS AND METHODS

Safety. The experimental protocols followed in the manuscript do not need special attention. The proper handling of lipopolysaccharide is well-documented.

Materials. Phorbol myristate acetate and carrageenan were obtained from Sigma (St. Louis, MO). WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was purchased from Takara Bio (Shiga, Japan). Polymorphoprep is a product of Axis Shield (Oslo, Norway). RPMI-1640 culture medium was purchased from GIBCO (Grand Island, NY), Ficoll−Hyaque was obtained from ICN Biomedicals, Inc. (Aurora, OH), and enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were products of Biosource International (Camarillo, CA).

Preparation of the Muscadine Grape Skin Extract. Muscadine grape skin powder was prepared after pressing grapes of the Ison variety for juice production and deseeding the grape pulp. The muscadine grape skins were then dried at 120 °F for 12 h in a forced-air pan dryer manufactured by Powell Manufacturing Company (Bennettsville, SC). The dried skins were ground in a Fitz Mill Comminutor Hammermill manufactured by the Fitzpatrick Company (Elmhurst, IL). A 10% (wt/vol) muscadine skin extract was then prepared by the addition of 50% (vol/vol) ethanol with periodic vortexing at room temperature for 2 h. The extract was then centrifuged to remove the precipitate. The contents of phenolics, expressed as gallic acid equivalents (*10*), and ellagic acid (*11*) were determined.

Isolation of Human Blood Monocytes. Human blood was collected in heparinized tubes, and the blood was diluted 2-fold with RPMI-1640 culture medium. The diluted blood (7 mL) was layered over 2.5 mL of Ficoll−Hypaque, and the tubes were centrifuged for 20 min at room temperature at 400*g* (*12*). Mononuclear cells were collected, washed, and cultured with RPMI-1640 culture medium containing 10% fetal calf serum, 50 units/mL of penicillin, streptomycin (50 μg/mL), and 2 mM L-glutamine.

Cellular Toxicity of the Muscadine Grape Extract. Human peripheral blood mononuclear cells were cultured with RPMI medium containing 10% fetal calf serum for 24 h in the presence of various concentrations of the muscadine grape skin extract. At the end of the incubation period, propidium iodide was added (final concentration of 25 μg/mL) and the percentage of dead cells was determined by flow cytometric techniques (*13*).

Cellular Release of Proinflammatory Cytokines. Peripheral blood mononuclear cells (1×10^5) were cultured in round bottom microtiter plates in 0.2 mL of culture medium containing 1 μg/mL *Escherichia coli* O111:B4 lipopolysaccharide and various concentrations of the muscadine skin extract. After 24 h, the cells were pelleted by centrifugation and the culture supernatants were collected and stored at −70 °C. The culture supernatants were thawed and assayed for IL-1β, TNF-α, and IL-6 using ELISA commercial kits. A standard curve using recombinant cytokines was included in each assay.

Superoxide Production by Neutrophils. Neutrophils were obtained from male Lewis rats, approximately 3 months of age, and were purified by density gradient centrifugation on Polymorphoprep (Axis Shield) according to the methods described by Tan and Berridge (*14*). The isolated neutrophils were incubated with muscadine skin extract for 1 h at 37 °C. Phorbol myristate acetate, dissolved in DMSO, was then added to the cells at a concentration of 100 ng/mL, and the cells were incubated for an additional hour at 37 °C. The production of superoxide radicals was measured by determining the reduction of the sulfonated tetrazolium salt, WST-1, spectrophotometically at 450 nm (*14*).

Carrageenan-Induced Edema. Male Sprague−Dawley rats (150−175 g) were fed either normal rat chow or rat chow supplemented with 5% (wt/wt) muscadine skin powder for 2 weeks. On day 15, the rats were injected with 0.5 mg of carrageenan into the subplantar region of the left hind paw while the contralateral hind paw received saline (*15*). Hind paw volumes (edema) were measured plethysmographically by displacement of mercury prior to and at 3 h postadministration of carrageenan. Swelling was determined by subtracting the volume of the right hind paw (saline control) from that of the left (carrageenan injected).

Statistical Analysis. Data are presented as the means ± standard errors of the mean (SEM). To determine statistical significance ($P < 0.05$) between groups, data were analyzed by either Student's *t*-test or by one-way analysis of variance and Duncan's multiple range test.

RESULTS

The antiinflammatory activity of muscadine grape skins was evaluated in this study. After the skins were dried, pulverized, and extracted (10% wt/vol) with 50% ethanol, the phenolic and ellagic acid contents of the extracts were determined. The phenolic content was 2.1 ± 0.1 mg/mL, and the ellagic acid content was 67 ± 5 μg/mL. To examine the possible toxic effect of the muscadine grape skin extract, human peripheral blood mononuclear cells were cultured with either the extract or the ethanol for 24 h, and the percentage of dead cells was determined by propidium iodide positive staining ascertained by flow cytometry. The highest concentration of ethanol (1% ethanol) was associated with the death of 3.5% of cells. Muscadine grape skin extract was associated with a concentration-dependent increase in cell death, ranging from 3.8% for a 1:400 dilution of the extract to 8.7% for a 1:100 dilution of the extract. Higher concentrations of the extracts produced greater cell death; therefore, experiments employed dilutions of muscadine grape skin extracts ranging from 1:400 to 1:100.

The biological properties of the muscadine extracts were examined in two different assays: (i) the release of cytokines by lipopolysaccharide-activated peripheral blood mononuclear cells and (ii) the production of superoxide free radicals in phorbol myristate acetate-activated neutrophils. The effect of the muscadine grape skin extract on the release of proinflammatory cytokines by activated peripheral blood mononuclear cells was initially investigated. In these experiments, the muscadine grape skin extracts were added at the same time as the lipopolysaccharide. As illustrated in **Figure 1a**, the muscadine skin extract produced a concentration-dependent inhibition in the release of TNF-α. A 1:200 dilution of the muscadine skin extract inhibited the release of TNF-α by approximately 15% while a 1:100 dilution produced nearly a 50% reduction. The muscadine skin extract was even more potent in inhibiting the cellular release of IL-1β (**Figure 1b**). A 1:400 dilution inhibited IL-1β release by approximately 60%; this same concentration of extract produced no effect on the release of TNF-α in this experimental system. Furthermore, 1:200 dilution of the muscadine grape skin extract inhibited the release of IL-1β by 93%. The pattern of inhibition of the muscadine grape skin extract on the release of IL-6 was somewhat similar to that observed for TNF-α (**Figure 1c**). When the cells were preincubated with the muscadine extract 2 h prior to the addition of lipopolysaccharide, a similar pattern of inhibition of TNF-α, IL-1β, and IL-6 release was observed (data not shown).

As illustrated in **Figure 2**, a concentration-dependent inhibition of superoxide production was observed. A 1:100 dilution of the extract inhibited superoxide production by approximately 65%. For comparison, aspirin, a known inhibitior of superoxide production, was employed as a positive control. Asprin, at a concentration of 100 μg/mL, inhibited superoxide production by approximately 70% (absorbance was 0.19 ± 0.03).

As a follow-up to the in vitro cellular data, an in vivo experiment was performed to determine the antiinflammatory activity of muscadine grape skin powder. In this experiment, the muscadine skin powder was fed to rats as opposed to an ethanol extract employed in the previous experiments. Male Sprague−Dawley rats were fed either normal rat chow or rat

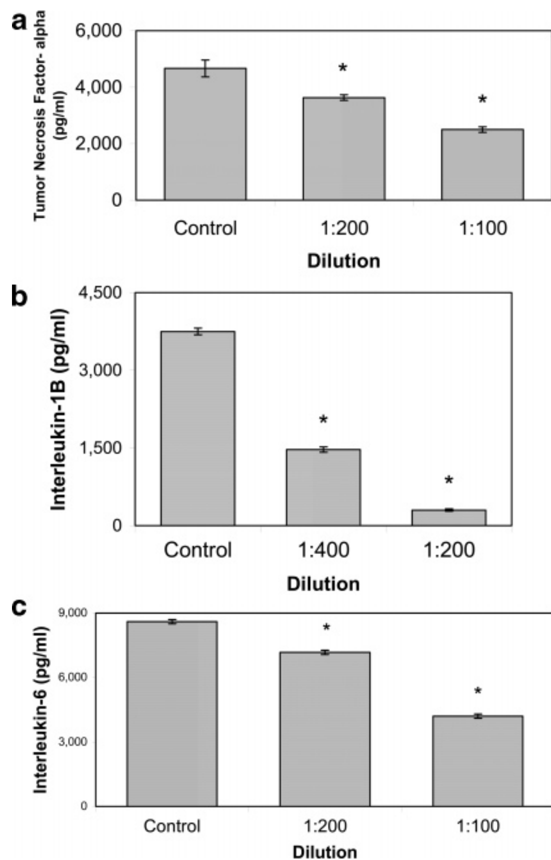

Figure 1. Muscadine grape skin extract-induced inhibition of cytokine release from human mononuclear cells. Cells were incubated with 1 μg/mL *Escherichia coli* O111:B4 lipopolysaccharide and various dilutions of the muscadine grape skin extract. After 24 h of incubation, the content of TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) was determined by ELISA. Control cultures were incubated with 1% ethanol. Results represent means ± SEM of three separate determinations. *$P < 0.05$ when compared to control cultures.

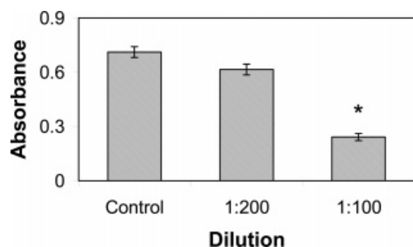

Figure 2. Effect of muscadine grape skin extract on the production of superoxide by activated neutrophils. Neutrophils were preincubated with various dilutions of the muscadine skin extract for 1 h at 37 °C while control cultures were incubated with 1% ethanol. The cells were then stimulated with phorbol myristate acetate (100 ng/mL), and production of superoxide radicals was determined after 1 h of incubation. Results represent means ± SEM of three separate determinations. *$P < 0.05$ when compared to control cultures.

chow supplemented with 5% (wt/wt) muscadine grape skin powder for 2 weeks. On day 15, carrageenan was injected into the subplantar region of the left hind paw. Hind paw volumes (edema) were measured by displacement of mercury prior to and at 3 h postadministration of carrageenan. As illustrated in **Figure 3**, animals previously ingesting muscadine skin powder for 2 weeks had significantly less paw edema than controls. These results demonstrate that powdered muscadine grape skin ingestion can affect the in vivo inflammatory cascade.

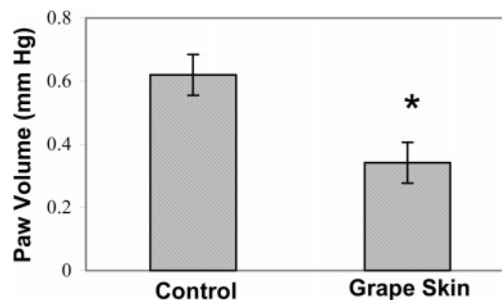

Figure 3. Effect of muscadine grape skin powder on carrageenan-induced paw edema. Rats were fed either normal rat chow or rat chow supplemented with 5% muscadine skin powder for 2 weeks. On day 15, the rats were injected with carrageenan and edema measured plethysmographically 3 h later. Results represent means ± SEM taken from eight rats. *$P < 0.05$ when compared to control rats.

DISCUSSION

Phytochemicals may affect numerous mediators that are involved in the inflammatory process. For example, the production of superoxide and the release of cytokines are important factors in both acute and chronic inflammatory states. In this regard, the production of superoxide by neutrophils is thought to be partially responsible for the local damage observed in chronic inflammatory diseases (*16*). IL-1β promotes the destruction of cartilage in rheumatoid arthritis (*17*) while TNF-α, aside from other functions, induces the production of IL-1β (*17*). Antibodies to TNF-α are employed therapeutically to inhibit the progression of joint damage in rheumatoid arthritis (*18*). IL-6 is also a proinflammatory cytokine that plays an important role in autoimmune disease and chronic inflammatory proliferative disease (*19*). The results presented in this communication demonstrate that muscadine grape skin extract has the capability to interfere with the inflammatory process at several different steps in the pathway. It is interesting to note that the muscadine grape skin extract produced a slightly greater cell death than ethanol controls (8.7% for a 1:100 dilution of the extract vs 3.5% for the ethanol control). This effect was quite small when compared to the decrease observed in the production of superoxide by neutrophils and the release of cytokines by human peripheral blood monocytes.

Muscadine grapes have extremely high antioxidant capacity (*1*, *4*, *8*). It has been known for many years that oxygen radicals facilitate the lipopolysaccrahide-mediated release of cytokines (*20*), and numerous studies have demonstrated that a wide variety of antioxidants inhibit the monocyte (macrophage) release of TNF-α, IL-1β, and IL-6 (*20*−*22*). In addition, oxygen radicals are important contributors to carrageenan-induced paw edema (*23*); superoxide dismutase [as presented as the poly(ethylene glycol) conjugate] decreases the degree of carrageenan-induced edema. Thus, the antiinflammatory properties of muscadine grape skin documented in this study can be, in part, a direct result of the antioxidant constituents of the grape.

It is quite interesting to note that the effect of muscadine grape skin extract on the release of proinflammatory cytokines is similar to that observed with SKF-86002 (*24*). This drug inhibited the release of IL-1β to a much greater extent than it inhibited the release of TNF-α and IL-6 from heparinized human whole blood (*24*). Interestingly, SKF-86002 is an inhibitor of 5-lipoxygenase and cyclooxygenase, suggesting that muscadine grape skin extracts may also target these enzymatic activities.

While this study demonstrated that the whole muscadine grape skin fraction has antiinflammatory properties, there are reports in the literature indicating that specific constituents of grapes

possess similar properties. Procyanidins from grape seeds (oligomeric catechin fraction) have been shown to inhibit superoxide generation in neutrophils (*25*). Similarly, intraperitoneal injection of grape seed proanthocyanidins decreased both the extent of paw edema in rats administered carrageenan and the content of TNF-α in the paw exudates (*26*). Gallic acid, catechin, quercetin, and genistein have been shown to inhibit TNF-α release in lipopolysaccharide and interferon-γ-stimulated macrophages (*27*). At the present time, the biological effects of muscadine grape skin powder cannot be attributed to any one constituent of the fruit.

The results presented in this communication suggest that muscadine grape skins may influence the inflammatory process in humans. Currently, the fresh fruit is commercially available for only 2 months a year while a significant fraction of the muscadine crop is juiced for wine production. After juicing, the muscadine pomace is usually treated as waste material with practically no commercial value. It is therefore reasonable to suggest that this pomace fraction can be processed for the value-added nutraceutical market for both year round consumption and possible human health benefits.

ABBREVIATIONS USED

TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant potential; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

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